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13. ABSTRACT ***(Maximum 200 Words)***

These studies address the question of how abnormal stromal-epithelial interactions affect the progression of cancer cells. Our studies in mouse mammary gland reveal that ionizing radiation, a known human breast carcinogen, elicits rapid and persistent global changes in the tissue microenvironment. If the microenvironments induced by carcinogens can shape the features and frequency of neoplastic phenotypes, then the carcinogen 'fingerprint' may be envisioned as being built by first laying a foundation of genotypic alterations that expand in the context of a microenvironment that is the result of alterations in stromal and epithelial phenotypes. The current studies are intended to test the hypothesis that *carcinogen-induced changes in the microenvironment constitute a third class of carcinogenic action distinct from those leading to genomic damage or proliferative advantage.* The long-term goal of this research is to determine whether definition of carcinogen-induced microenvironments predicts neoplastic features or frequency. Understanding this aspect of carcinogenesis is important since certain microenvironment alterations might be suitable for therapeutic intervention, which in turn could provide the mean to modify cancer progression.

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INTRODUCTION

We have proposed the hypothesis that *carcinogen-induced changes in the microenvironment constitute a third class of carcinogenic action distinct from those leading to mutation or proliferative advantage.* Carcinogen-induced microenvironments are postulated to increase the number or susceptibility of epithelial cells to transformation, exert a selective force on initiated cells and/or are conducive to progression. If the microenvironment induced by carcinogens can shape the features and frequency of neoplastic phenotypes, then the carcinogen 'fingerprint' may be envisioned as being built by first laying a foundation of genotypic alterations that expand in the context of a microenvironment that is the result of carcinogen-induced phenotypic change. Understanding this aspect of carcinogenesis is important since certain microenvironment alterations might be amenable to modulation, which in turn could provide the means to modify cancer progression. The proposed studies are intended to obtain further evidence for this hypothesis.

We have studied the effects of a known breast carcinogen, ionizing radiation, on the microenvironment of the mouse mammary gland. We have showed that mouse mammary gland extracellular matrix undergoes rapid and global remodeling that includes the novel expression of tenascin and collagen type III. This remodeling is mediated by the activation of the multipotent cytokine, transforming growth factor-β1 (TGF-β), a potent regulator of both epithelial and stromal function. We have shown that activation can be detected at doses as low as 0.1 Gy and that blocking TGF-β with neutralizing antibodies inhibits radiation-induced extracellular matrix remodeling, providing functional confirmation of TGF-β activity. Based on these studies, we concluded that exposure to a carcinogen such as radiation can elicit persistent changes in gene expression by non-initiated cells.

By creating chimeric mammary glands consisting of normal or irradiated mammary epithelium in normal or irradiated stroma, we found that the irradiated stroma impedes epithelial maturation. The first aim of the present grant is to test whether radiation-induced TGF-β activity regulates this phenotype. The effect of the irradiated stroma may relate to the well-documented age dependence of radiogenic breast cancer. For example, if radiation-induced microenvironment delays the development of the gland in differentiating past a critical check point, then the size or sensitivity of the carcinogen-susceptible population may be increased. Alternatively, radiation-induced TGF-β may be a selective force that allows expansion of initiated cells resistant to TGF-β. To test whether preneoplastic cells progress more readily in an abnormal stroma, we propose in aim two to create chimeric glands consisting of preneoplastic epithelium in normal versus irradiated stroma.

We predicted that, given the known age dependence of radiogenic mammary cancer in both mice and women, the character of the microenvironment would change as a function of both radiation exposure and mammary development. Our third objective is to compare the radiation-induced microenvironment of adult and immature mice, with particular attention to the expression and activity of TGF-β. The regulation of TGF-β activation and activity in vivo is not well-understood. We have begun this study by examining the effect of development, hormonal status, and differentiation on TGF- β activation.

Year 4 STUDIES and FINAL REPORT

Aim 1: Determine the role of TGF-β in the inhibition of mammary gland development by irradiated stroma by using neutralizing antibodies to knockout TGF-β activity during outgrowth.

We have demonstrated that remodeling of the irradiated mouse mammary gland microenvironment is mediated in part by TGF-β [1 Ehrhart, 1997]. TGF-β is an important regulator of differentiation, proliferation and extracellular matrix composition. It has been postulated to play both a positive and negative role in cancer development and progression [2], which suggests that determining its physiological regulation and activity in particular tumors may provide interesting targets for therapy [3]. We have published a review of the role of TGF-β during mammary gland development in **Breast Cancer Research** (appendix I) based on our research supported by DOD [4].

TGF-β is secreted as a latent complex that is unable to bind to TGF-β receptors until the biologically active 24-kD mature TGF-β is released; this activation is considered to be the critical regulatory event for TGF-β function. Radiation exposure elicits rapid and persistent activation of TGF-β *in vivo*. We postulated that aberrant TGF-β activation by ionizing radiation affects mammary gland development and neoplastic progression by perturbing the balance between the stroma and epithelium. Prior to examining the effect of developmental status on the response to radiation, we determined the distribution and abundance of active TGF-β in Balb/c mice during normal mammary development. Using an immunostaining protocol that preserves endogenous latent TGF-β and antibodies that discriminate between latent and active TGF-β, we determined that in normal adult mammary gland latent TGF-β is abundant but active TGF-β is restricted to epithelial structures [5] (an example is shown inAppendix I).

To evaluate the consequences of TGF-β activity during development, we proposed to use neutralizing antibodies. This has proven useful for determining the immediate consequences of TGF-β activity in irradiated tissue [1]. However chronic administration of exogenous antibodies can have immune effects. Thus we turned to a mouse model that was made available to us after the inception of this proposal in which the TGF-β1 gene has been deleted. TGF-β null mutant mice have been very informative in demonstrating that the TGF-β isoforms perform distinct functions in vivo even though they share sequence homology and receptors [6]. This specificity may in part be due to distinctive LAP sequences that dictate both localization and susceptibility to activation [7]. Mice in which TGF-β is deleted by targeted gene knockout die at weaning from grossly inflamed tissues [8], which thus precludes analysis of postnatal mammary development. However, TGF-β heterozygotes exhibit significantly compromised TGF-β levels (10-30% of wild type), indicating that endogenous TGF-β regulates, directly or indirectly, its own production and/or stability [9]. We evaluated TGF-β heterozygotes to determine whether reduction of endogenous TGF-β alters mammary growth. These animals provide a new model in which to test the significance of TGF-β in the irradiated stroma.

Effect of TGF-β1 Allelic Loss During Development

As we reported in year 3, duct elongation is 2-fold greater in mammary glands of TGF-β1 null heterozygotes examined during puberty (6 weeks of age) than in wild types. Moreover, we found that PCNA, a marker of cell cycle status, was elevated approximately 3-fold in the epithelial cells of endbuds, proliferative structures at the end of elongating ducts, and more than 2-fold in ducts of

TGF-β1 null heterozygotes. This year we looked at apoptosis in the endbuds, since this process is regulated by TGF-β1 in other tissues. Apoptosis is required to hollow out the endbud to form a duct. However, apoptosis was found to be unchanged in TGF-β1 null heterozygotes compared to wild types (2.2 ± 0.4% versus 2.8 ± 0.4%). This is consistent with the normal morphology of the ducts in TGF-β1 null heterozygotes.

The developmental role of TGF-β1 was extended this year to adult nulliparous mice. The morphology of the ducts in TGF-β1 null heterozygotes are not detectably different from wild types. Indeed, the area occupied by ducts in sections of mammary glands of TGF-β1 null heterozygotes are not significantly different (t-test, P=0.04) to those of wild type littermates. However, in dynamic terms, the TGF-β1 null heterozygotes are quite different from wild types (Table 1). In estrous, TGF-β1 null heterozygotes have a significantly higher proliferative index and a significantly lower index of apoptosis. However there is a much higher apoptotic index in TGF-β1 null heterozygotes at proestrous than in wild types. This could be the result of homeostatic compensation for the high proliferation and low apoptosis found in TGF-β1 null heterozygotes, explaining why TGF-β1 null heterozygotes do not exhibit hyperplasia.

TABLE 1 : Proliferation and Apoptosis in Adult Nulliparous TGF-β1 Null Heterozygote and Wild Type Mice

INDEX	**GENOTYPE**	
Estrus Status	**Heterozygote**	**Wild Type**
Proliferation (% PCNA positive) [a]		
Proestrous	0	0.15 ± 0.05
Estrous	1.90 ± 0.60	0.55 ± 0.25*
Diestrous	0.35 ± 0.05	0
Apoptosis (% Pyknotic Nuclei) [b]		
Proestrous	0.67 ± 0.03	0.26 ± 0.04*
Estrous	0.22 ± 0.01	0.48 ± 0.03*
Diestrous	0.59 ± 0.03	0.44 ± 0.05

[a] Paraformaldehyde-fixed frozen sections were stained with a FITC-conjugated antibody to PCNA. At least 1500 cells from 2 animals were scored for presence of PCNA immunoreactivity. Mean ± standard error shown.
[b] H&E-stained paraffin sections were scored for pyknotic figures. At least 6000 cells from 2 animals were counted. Mean ± standard error shown.
*Significantly different using Student 2-tailed t-test.

TGF-β1 Allelic Loss Leads to Elevated Proliferation After Both Estrogen and Progesterone Replacement

Hormone replacement after ovariectomy in TGF-β1 null heterozygotes and wild types is necessary to determine whether TGF-β1 mediates the response of mammary epithelium to estrogen and/or progesterone. The level of proliferation in TGF-β1 null heterozygotes in ovariectomized mice is dramatically higher than that in wild types when administered estrogen and progesterone for 3 days and for 9 days (Table 2). Proliferation after 1 day of hormone replacement was at control (saline) levels in both TGF-β1 null heterozygotes and wild types. Administration of both hormones is analogous with estrous, with its relatively high levels of estrogen and progesterone, in which the loss of one TGF-β1 gene also led to an elevated index of proliferation. The proliferative index in ovariectomised mice supplemented with estrogen alone for 3 days was elevated compared to TGF-β1 null heterozygotes, but not after 9 days. Thus, TGF-β1functions most effectively to restrain proliferation when both hormones are present. We found that proliferation in mice that were given progesterone had indices of proliferation similar to control mice.

TABLE 2: PCNA Labelling Index of Epithelial Cells In Ovariectomised and Hormonally Treated TGF-β1 Null Heterozygous and Wild Type Mice

TREATMENT	% PCNA positive[b]	
DAY 3	**Heterozygote**	**Wild Type**
Saline	0.3 ± 0.05	0.1 ± 0.05
E	3.3 ± 0.20*	0.3 ± 0.10
P	0.2 ± 0.00	0.3 ± 0.00
E + P	7.7 ± 4.2*	0.5 ± 0.15
DAY 9		
Saline	0.3 ± 0.05	0.1 ± 0.0
E	0.2 ± 0.10	0.3 ± 0.10
P	0.3 ± 0.10	0.2 ± 0.0
E + P	3.6 ± 0.6*	0.5 ± 0.15

[a]Mice were ovariectomized 17 days before commencement of hormone replacement. For hormone replacement, 1 mg of progesterone and/or 1 mg estradiol in saline was administered daily.
[b]Paraformaldehyde-fixed frozen sections were stained with a FITC-conjugated antibody to PCNA. At least 1500 cells from 2 animals were scored for presence of PCNA immunoreactivity. Mean ± standard error shown.
* Indicates significant difference by two-tailed Student t-test.

These data provide valuable insight into the regulation of TGF-β1 activity during mammary gland development and proliferation. As a result of these studies, we believe that the TGF-β1heterozygote could be used to advantage to understand whether TGF-β1 is a significant factor that contributes to the promoting action of irradiated stroma on neoplastic potential. However the mice are currently only available in the C57/bl6 mouse strain. Future use will require that the knockout be crossed into

the Balb/c background.

Aim 2: Determine the effect of sham versus irradiated fat pads on the carcinogenic potential of the COMMA1-D mammary epithelial cell line harboring defective p53 genes.

These studies were the main objective of the proposal. We recently published these studies in **Cancer Research** (Appendix II) demonstrating that the irradiated stroma affects the neoplastic potential of unirradiated COMMA-D mammary epithelial cells. These data were presented by the PI in platform presentations at Mammary Gland Biology Gordon Conference and Workshop on the Role of Tissue Architecture in Breast Cancer, Washington, DC in 1999 and at the Radiation Research Society meeting in Albuquerque, New Mexico in 2000. They were reported as poster presentations at the International Congress on Radiation Research in Dublin, Ireland in 1999and a the Era of Hope meeting in Atlanta, Georgia in 2000.

These data are the basis for new funding in 2000 by the California Breast Cancer Research Program entitled "Role of p53 in the Irradiated Stroma and Mammary Carcinogenesis". In this proposal we will implement a new model system using Babl/c *p53null* epithelial cells in order to study the action of radiation on the stroma in more detail. We will transplant *p53null* epithelial to wildtype stroma to test the precise radiation exposure conditions under which we can observe a statistically significant effect on tumorigenic potential as a function of radiation dose, dose rate, systemic vs local effects, and hormonal status. These studies will determine whether the action of p53 is specifically produces a preneoplastic population susceptible to promotion by irradiated stroma.

Aim 3: Define the radiation-induced microenvironment as a function of mammary gland developmental status.

Dr. Rhonda Henshall joined the lab in April, 2000 to undertake the studies for this objective. She has completed tissue collection from mice irradiated as a function of age (3, 5 and 8 weeks old) for wholemount and histological analysis. There was no evidence of radiation effects on hormonal status in our previous studies of adult mice (12 w.o.), but it is possible that establishment of ovarian function of young mice might be more susceptible to irradiation. Morphological markers of estrogenic action were measured using cytosmears of the vaginal epithelium and uterus weight and status in irradiated animals were compared to age-matched controls. No difference was found between the sham-irradiated groups (1 hr and 1 day) and those irradiated 1 hr, or 1, 3, and 7 days before sacrifice. Wholemounts were analyzed as a function of time (1 hr, 1 d, 3 d and 7 d; n=6/time point) following whole body irradiation with 4 Gy for gross morphological endpoints such as endbud number and size, percent of fat pad filling, and width of ducts. Again, no significant differences were observed between sham-irradiated animals and irradiated animals in any of the gross morphological features. Taken together, these data suggest that gross ovarian function is intact following this dose of ionizing radiation.

Also, as reported last year, we evaluated the physiological mechanisms of TGF-β activation and the consequences of its activity during mammary gland development. Our manuscript describing studies of the physiological regulation of TGF-β activation in normal murine mammary gland reports the novel observation that activation is highly restricted and is differentially regulated by differentiation and estrus [5]. Expansion of these studies to determine the fate and features of TGF-β positive epithelial cells were funded in 1999 by the California Breast Cancer Research Program. The postulated role of TGF-β as a key regulator of normal mammary proliferation will be examined using specific hormonal manipulations and transgenic mouse models.

KEY RESEARCH ACCOMPLISHMENTS

This funding has supported experiments leading to several important and novel observations:

1. *The abnormal stroma created by radiation exposure promotes the neoplastic potential of COMMA-D mammary cells.*
2. *The results above has led us to propose that a third action of carcinogens, distinct from inducing genomic change or affecting proliferation, is to modify tissue microenvironments in such a way as to promote neoplastic potential*
3. *In regards to the role of TGF-β, we have discovered that activation is spatially restricted within the epithelium*
4. *Also that TGF-β production and activation are differentially regulated by the ovarian steroid hormones, estrogen and progesterone.*
5. *Studies using TGF-β knockout mice have shown that endogenous TGF-β suppresses mammary proliferation and support the evidence of hormonal regulation of activation.*

REPORTABLE OUTCOMES

Publications

PEER REVIEWED

Barcellos-Hoff, M.H. & Ravani, S.A.: Irradiated mammary gland stroma promotes the expression of tumorigenic potential by unirradiated epithelial cells. **Cancer Res**, 60:1254-1260, 2000.

Barcellos-Hoff, M.H. & Shyamala, G.: Latent TGF-β activation is regulated by estradiol and progesterone during mammary gland development. **Submitted, 2000.**

Ewan, K.B., Shyamala, G., Wakefield, L. & Barcellos-Hoff, M.H.: Endogenous TGF-β mediates hormone-induced proliferation in mammary gland. **Submitted, 2000.**

INVITED REVIEWS

Barcellos-Hoff, M.H.: The potential influence of radiation-induced microenvironments in neoplastic progression. **J. Mammary Gland Biol. Neoplasia**. 3(2):165-175, 1998.

Barcellos-Hoff, M.H.: How do tissues respond to damage at the cellular level? The role of cytokines in irradiated tissues. **Radiat. Res.** 150:S109-120, 1998.

Barcellos-Hoff, M.H. and Ewan, K. B. : Developmental regulation of transforming growth factor β1. **Breast Cancer Research**. 2:92-100, 2000

Park, C., Bissell, M.J. and Barcellos-Hoff, M.H.: The role of the microenvironment in the malignant phenotype: Critical role of cytokines and integrin signaling. **Molecular Medicine Today** In press. 2000

Abstracts

Barcellos-Hoff, M.H. and S.A. Ravani. Irradiated Mammary Gland Stroma Promotes the Tumorigenic Potential of Unirradiated Epithelial Cells. DOD Era of Hope, Atlanta, GA, June, 2000.

Barcellos-Hoff, M.H., K.B. Ewan, G. Shyamala, Hormonal Regulation of TGF-β During Mammary Gland Development. California Breast Cancer Research Symposium, September, 1999.

Barcellos-Hoff, M.H. and S.A. Ravani, Irradiated Stroma is Conducive to Neoplastic Progression of p53 Mutant Mammary Epithelial Cells. International Congress in Radiation Research, Dublin, July, 1999.

Barcellos-Hoff, M.H. and Shyamala, G. Regulation of Transforming Growth Factor-β1 Production and Activation During Mammary Gland Development. Epithelial Cell Biology 98, Oxford, England; September, 1998.

Barcellos-Hoff, M.H. The Irradiated Mammary Stroma is Conducive to Neoplastic Progression of Mammary Epithelial Cells Harboring Defective p53. Radiation Research Society Meeting, Louisville, KY; May, 1998.

Barcellos-Hoff, M.H. and Shyamala, G. TGF-β Activation is Regulated during Mammary Gland Growth and Differentiation. NIH Meeting on Breast Development, Physiology and Cancer, June 25–28, 1997.

Barcellos-Hoff, M.H., S. Ravani, and M.T. Finnegan. Carcinogen-Induced Microenvironments in Breast Cancer: A Role for Transforming Growth Factor-ß1. DOD Breast Cancer Research Investigators' Meeting, Washington, D.C., Oct. 31-Nov. 3, 1997.

Presentations

"Dynamic Remodeling of the Microenvironment by Radiation and Its Role in Mammary Carcinogenesis" Radiation Research Society Annual Meeting, Albuquerque, NM, May 3, 2000.

"Induction of Mammary Gland Carcinogenesis by Irradiation of the Stroma" Workshop on the Role of Tissue Architecture in Breast Cancer, Washington, DC, September 15, 1999.

"How Do Tissues Respond to Damage at the Cellular Level?" NASA Investigators Meeting, Brookhaven, NY, June 13, 1999.

"The Role of the Irradiated Microenvironments in Mammary Epithelial Neoplastic Progression" Gordon Conference on Mammary Gland Biology, New London, NH, June 9, 1999.

"Role of Cytokines in Normal Tissue Damage", Biomed Concerted Action on Predictive Assays, Canne, France, October 11, 1998.

"Regulation of Transforming Growth Factor-β Production and Activation During Mammary Gland Development", Epithelial Cell Biology '98, Oxford, England, September 14, 1998.

"Mechanisms and Consequences of TGF-β Activation in the Mammary Gland" University of Manchester, England, September 11, 1998.

"Extracellular Signalling: The Control of Cytokine Action" Radiation Research Refresher Course, Louisville, KY, April 29, 1998.

"The Role of Biomolecules in Breast Cancer Diagnostics" 20th Annual Engineering Industrial Liaison Conference, University of California, Berkeley, March 11, 1998.

"Mechanisms and Consequences of TGF-β Activation in Mammary Gland" Keystone Breast and Prostate Cancer Symposia, Copper Mountain, CO, February 23, 1998.

"TGF-β Activation During Mammary Gland Development and Carcinogenesis" Laboratory of Chemoprevention, National Cancer Institute, Dr. Anita Roberts (host) Bethesda, MD, November 3, 1997.

"Carcinogen-Induced Microenvironments in Breast Cancer: A Role for Transforming Growth Factor β1" DOD Era of Hope for Breast Cancer, Washington, D.C., November 2, 1997.

"Definition of Ionizing Radiation Induced Tissue Microenvironments and Its Influence on Neoplastic Progression", Sponsored Jointly by Environmental Toxicology and Department of Biology, University of California, Dr. Manuela Martins-Green (host), Riverside, CA, October 16, 1997.

"Mechanisms and Consequences of TGF-β Activation *In Vivo*", Laboratory of Human Carcinogenesis, National Cancer Institute, Dr. William Bennett (host), Bethesda, MD, September

17, 1997.

"Latent TGF-β Activation *In Situ*: Mechanisms and Consequences", Gladstone Foundation, University of California, Dr. David Dichek (host), San Francisco, CA, June 30, 1997.

"Mechanisms and Consequences of TGF-β Activation", Fibrogen, Dr. Patricia Segarini (host) Santa Clara, CA, June 3, 1997.

"Radiation-Induced Activation of TGF-β: Mechanisms and Consequences", Department of Virology, University of Freiburg, Dr. Georg Bauer (host), Freiburg, Germany, April 2, 1997.

3. Patents: None

4. Other

Funding based (in part) on research supported by this award

Hormonal Regulation o f TGF-β During Mammary Development, Principal Investigator, CA-BCRP $451,011 (direct, 1998-2001). We propose to use confocal and digital microscopy to correlate spatial and temporal patterns of TGF-β activation with hormone receptors, DNA synthesis, apoptosis and differentiation. We will use transgenic mice to misregulate this expression in specific ways so that complex tissue interactions and morphogenesis can be followed in vivo. This provides the opportunity to examine cause-and-effect relationships between TGF-β and hormone responses in mammary gland. In this proposal we seek to identify the characteristics of both TGF-β negative and positive populations and determine their fate. We will then functionally test two hypotheses: First, that TGF-β is regulated by progesterone will be evaluated using progesterone receptor knockout animals. Second, that TGF-β activity mediates mammary hormone response will be assessed using targeted depletion of TGF-β.

Mechanisms of Tissue Response to Low Dose Ionizing Radiation Exposures: Bioinformatic Tools for Multiparametric Image Analysis, Principal Investigator, DOE, $1,248,000 (direct, 1998-2001). This proposal will examine the dose, tissue and temporal dependence of radiation induced proteins, identify the underlying mechanisms by which tissue responses are implemented and evaluate their relevance in terms of impact on human cancer risk. Murine mammary glands from transgenic and various genetic backgrounds and 3-dimensional models of human mammary epithelial cells will be used to identify common cross-species features of radiation response. We will use digital fluorescence microscopy to map and quantify radiation effects in these models. In order to increase the sensitivity, throughput and accuracy of image analysis we will develop a bioinformatics framework. Image acquisition, annotation, analysis will be integrated with an image database that registers information about multiple targets, positional references and morphological features. This multidisciplinary and innovative approach will enable construction of phenotype databases necessary to identify critical biological responses to low dose radiation exposure that can be used in computational models of radiation risk.

Role of p53 in the Irradiated Stroma and Mammary Carcinogenesis, Principal Investigator, CA-BCRP 2000-2002, $336,876 total. To further study the role of stroma in radiation carcinogenesis, we will establish a new model using mammary cells from *p53* ^{null} Balb/c mice developed by our collaborator, Dr. D. Joseph Jerry (University of Massachusetts, Amherst). We will examine radiation dose, dose-rate, and hormonal status as means of evaluating the contribution of the stroma to carcinogenesis. These experiments will establish a robust model system in which future studies will determine which factors from the irradiated stroma are

critical to its tumorigenic potential.

CONCLUSIONS

Transforming growth factor-β1 (TGF-β) inhibits proliferation of mouse and human mammary epithelial cells maintained in cell culture, but as yet direct evidence for this inhibition by endogenous TGF-β has been lacking. Furthermore, since biological activity of TGF-β is controlled by its secretion as a latent complex formed by non-covalent association with latency associated peptide (LAP), the contribution of spatial and temporal constraints on its extracellular activation have not been defined. To localize when and where activation occurs *in situ,* we used dual immunofluorescence detection of anti-LAP relative to anti-chNTGF-β to identify latent versus active TGF-β respectively and digital image analysis to measure their relative expression. Certain epithelial cells exhibited low LAP and high chNTGF-β intensity, which is indicative of latent TGF-β activation. These cells were dispersed throughout the epithelium at certain developmental stages: in endbuds during puberty, at estrus in adult nulliparous mice, and in early pregnancy. We now show that TGF-β haploid genotype leads to accelerated mammary development: duct elongation was accelerated in heterozygotes versus wildtype mice and proliferating cell compartment was increased up to 4 fold in the endbud. Although grossly normal, mammary epithelium from adult, nulliparous animals also exhibited increased proliferation. Taken together, these data provide evidence that endogenous TGF-β is a growth inhibitor during mammary gland development, and that latent TGF-β activation, and thus TGF-β biological activity, is highly regulated *in situ.*

Although the particular aspect of the irradiated mammary microenvironment that is stimulating COMMA-D tumorigenic conversion is unknown, the ability of radiation to induce TGF-β activation may indeed play a role [7, 10]. We have argued that TGF-β's contradictory role in cancer is because conversion from TGF-β sensitive to TGF-β resistant during tumor progression is a critical juncture in establishing malignant behavior of certain epithelia, like breast [3]. TGF-β has been identified as a cancer-promoting agent in wounds [11-13] and recent studies have implicated cyclosporin-induced TGF-β in the increased frequency of neoplasms following immunosuppressive therapy [14]. TGF-β also mediates phenotypic conversion of epithelial cells to vimentin-positive spindle cells [15]. Such a transition is observed in a transgenic keratinocyte model overexpressing TGF-β activity in which the frequency of benign papillomas is suppressed following chemical carcinogenesis, but progression to more malignant spindle cell carcinomas is stimulated [16]. In the SCp2 cell line, a subclone of late passage COMMA-D [17], TGF-β treatment stimulates the transition from non-tumorigenic keratin-positive cells to tumorigenic, vimentin-positive cells (Galosy, Barcellos-Hoff, Werb & Bissell, unpublished observations). Studies to determine whether the carcinogenesis promoting effect of radiation on stroma is due to TGF-β activation are underway.

Our hemibody irradiation data support the conclusion that radiation alters the local tissue microenvironment in a way that compromises the restraints imposed by normal stroma on initiated epithelial cells. A role for stroma early in neoplastic progression has also been suggested in hematopoeitic malignancies, which have been proposed to result from misregulation of adhesive properties by diseased or genetically aberrant stroma [18]. Conversely, the therapeutic benefit of α-interferon in chronic myeloid leukemia has recently been shown to be due in part to the re-establishment of cell-adhesion signals [19]. Greenberger and colleagues proposed a model of indirect γ-irradiation leukemogenesis based on co-cultures of heavily irradiated bone marrow stromal cell lines that selectively bound M-CSF receptor positive unirradiated hematopoietic progenitor cells resulting in selection of tumorigenic subclones (reviewed in [20]). Additional evidence that radiation

effects on stroma alter the behavior of neoplastic cells comes from studies of tumor bed effect, in which stroma that is heavily irradiated prior to tumor transplantation inhibits tumor growth but fosters metastatic behavior [21]. Such studies support the conclusion that radiation has general and persistent effects on stromal function that influences neoplastic progression.

Based on these data, we propose that radiation-induced microenvironments are evidence of an additional class of carcinogenic action, distinct from those leading to mutations or proliferation [22]. Studies in cell culture indicate that the frequency of morphological transformation can be modulated by restrictive conditions that select for preexisting cell variants [23] and that, conversely, normal cells may actively restrain the expression of the transformed cell phenotype [24]. In vivo studies by Zarbl and colleagues show that mammary tumors with *Hras*1 gene mutations from *N*-nitroso-*N*-methylurea treated rats arose from cells with preexisting *Hras*1 mutations that occurred during early development [25]. Thus, although clearly mutagenic in its own right, *N*-nitroso-*N*-methylurea exposure led to the expansion and neoplastic progression of *Hras1*-mutation containing populations. In our studies, radiation did not directly induce additional mutagenic events since the epithelial cells were unirradiated. We propose that a further action of carcinogens such as ionizing radiation is to modify paracrine interactions between the stroma and epithelium in a manner that affects the frequency with which previously initiated cells progress [26]. Carcinogen-induced microenvironments are not necessarily mutagenic or mitogenic *per se*. Rather, changes in the microenvironment may be conducive to neoplastic progression by disrupting normal cell inhibition of malignant behavior that is regulated through cell-cell contact, cell-ECM interactions and growth factor production. Thus, if ionizing radiation induces a microenvironment that modifies restrictive interactions, then progression may result just as it would if there additional mutations in the initiated cell. Alternatively, the microenvironment elicited by carcinogen exposure could create novel selective pressures that would affect the features of a developing tumor. Disruption of solid tissue interactions is a heretofore unrecognized activity of radiation as a carcinogen, and a novel avenue by which to explore new strategies for intervening in the neoplastic process.

These studies provide evidence that supports our hypothesis that the microenvironment elicited by carcinogen exposure are unique, and further that they may act to promote neoplastic progression. As a result, the possibility that microenvironments may be a future target for therapeutic intervention or cancer prevention gains credence (reviewed in [27], appendix III).

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Review

Transforming growth factor-β and breast cancer Mammary gland development

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Abstract

Transforming growth factor (TGF)-β_1 is a pluripotent cytokine that profoundly inhibits epithelial proliferation, induces apoptosis, and influences morphogenesis by mediating extracellular matrix deposition and remodeling. The physiologic roles of the action of TGF-β in mammary gland, indeed in most tissues, are poorly understood. In order to understand the actions of TGF-β, we need to take into account the complexity of its effects on different cell types and the influence of context on cellular responses. This task is further compounded by multiple mechanisms for regulating TGF-β transcription, translation, and activity. One of the most significant factors that obscures the action of TGF-β is that it is secreted as a stable latent complex, which consists of the 24-kDa cytokine and the 80-kDa dimer of its prepro region, called latency-associated peptide. Latency imposes a critical restraint on TGF-β activity that is often overlooked. The extracellular process known as activation, in which TGF-β is released from the latent complex, is emphasized in the present discussion of the role of TGF-β in mammary gland development. Definition of the spatial and temporal patterns of latent TGF-β activation *in situ* is essential for understanding the specific roles that TGF-β plays during mammary gland development, proliferation, and morphogenesis.

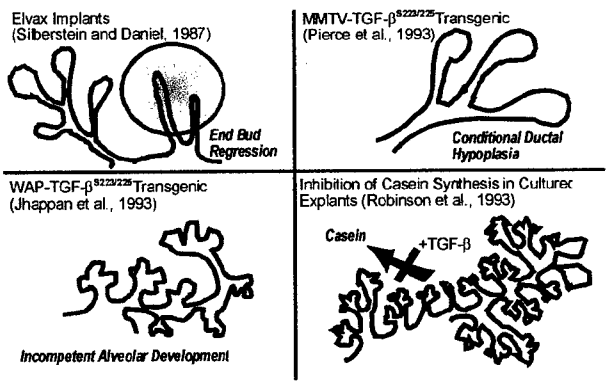
Keywords: transforming growth factor (TGF)-β_1, activation, latent, mammary, proliferation

Introduction

This review describes briefly mammary gland development and discusses the postulated role of TGF-β_1 in mammary gland development as evidenced by its effects in mammary epithelial cell culture and animal studies. TGF-β has been strongly implicated in the control of mammary epithelial growth [1] and in breast cancer cells [2,3]. Cell culture studies have also shown that the effects of TGF-β are pleiotropic, context-dependent, concentration-dependent, and can be indirect. As a consequence it is difficult to predict the endogenous action of TGF-β in mammary gland. However, mammary gland mouse models in which exogenous TGF-β or blocking antibodies are administered indicate that regulation of growth and morphogenesis is a potential function of TGF-β (Fig. 1). Transgenic models in which the activity of TGF-β is manipulated have confirmed this conclusion. Interpretation of these animal studies are confounded by the fact that the lack of knowledge about when and where TGF-β is active *in situ*, however. Preliminary studies from our laboratory have provided an indication of the complexity that awaits analysis of *in situ* activity.

ECM = extracellular matrix; LAP = latency-associated peptide; LTGF = latent transforming growth factor; MMTV = mammary mouse tumor virus; TGF = transforming growth factor; WAP = whey acidic protein.

Figure 1



Effects of transforming growth factor (TGF)-β in mammary gland. A variety of studies suggest that TGF-β contributes to morphogenesis, growth, and function in mouse mammary gland.

Mammary gland development

The seminal work of Daniel and coworkers [4••,5] focused attention on the role of TGF-β in mammary gland development soon after its characterization. The morphologic and functional development of the mammary gland is largely postnatal in both humans and mice (for review [6]). Under the effects of hormones of puberty, which begins just after weaning in mice and continues for several weeks (ie 3 weeks to 6–8 weeks of age), the mammary tree is established within an adipose stroma known as the fat pad. The epithelium, usually a simple epithelium consisting of one or two cell layers, is ensheathed in a fibrous stroma. This period of growth is characterized by a specialized morphologic unit called the end-bud, which is a multicellular, multilayered structure whose function is to extend the ductal epithelial tree to the boundaries of the fat pad. The pattern of the resulting tissue can be visualized in a whole-mount preparation and is often used as an end-point for evaluating the role of factors such as TGF-β in guiding morphology by measuring the extent of branching, the branching pattern by branch-point analysis, and the distribution of end-buds and their character, size, shape, and persistence. Once the ductal tree is established, repeated estrus cycles of ovarian hormones elicit further elaboration of the epithelium in some mouse strains, generating small lateral branch points. In a nulliparous animal approximately 10% of the tissue is epithelial.

Under the effects of hormones of pregnancy, a burst of growth and differentiation results in lobuloalveolar differentiation of the epithelium into functional secretory units that produce milk that is carried to the teat by large conducting ducts. The resulting fully functional gland is 90% epithelial. Upon weaning, a process of involution destroys the majority of secretory epithelium, leaving the ductal tree extant for repeated cycles of growth and differentiation.

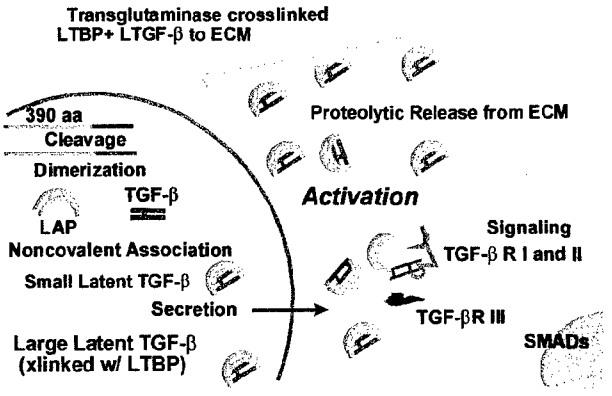
The remarkable capacity of the mammary epithelium to undergo development and differentiation provides a research model in which the factors that influence growth, morphologic patterning, proliferation, and differentiation can readily be explored. Furthermore, in mice epithelium can be transplanted to new stroma that is usually cleared of endogenous epithelium, which offers an exceptional research tool for studying the contribution of the stroma to the control of epithelial function and remodeling. Recombinations of transgenic and wild-type epithelia and stromas, and the ability to transplant intact fat pads to new hosts, allows determination of whether the source of a particular factor (ie different tissue compartments) affects the maturation of the mammary gland.

Transforming growth factor-β regulation and expression

There are three mammalian isoforms of TGF-β. All three isoforms bind to the same cell surface receptors and, in cell culture, often appear to elicit similar responses. For the purposes of this review we focus on the well-studied TGF-β_1 isoform, its localization in the mammary gland, its effects in cell culture models, and the consequences of manipulating its activity *in vivo* by various means. The activity of all TGF-β isoforms is restrained by secretion as latent complexes [latent transforming growth factor (LTGF)-β] [7]. (Regarding nomenclature, TGF-β refers to the active cytokine or its effects, whereas LTGF-β designates the latent form.) LTGF-β consists of the 24-kDa cytokine and a 80-kDa dimer of its prepro region called latency-associated peptide (LAP), which contains the signal sequence for secretion (Fig. 2). To our knowledge, all cells secrete LTGF-β, which underscores the importance of activation events associated with the release of TGF-β from LAP.

TGF-β message levels do not usually reflect protein production, or, more importantly, protein activity. For example, tamoxifen increases both LTGF-β production and activation without any effect on mRNA levels [2]. Differences in mRNA translation are in part due to a 5′-untranslated region stem-loop that binds a regulatory protein, which can result in increased protein production by factors of 10 without changes in mRNA levels [8]. In addition, protein assays are complicated by the fact that tissue extraction protocols activate the endogenous LTGF-β [9]. Because the biologic activity of TGF-β is controlled by its release from the latent complex, elevated expression of latent complex is not likely to have biologic consequences, whereas increased activation, even without changes in synthesis, will profoundly affect physiologic events [10]. The importance of activation is supported by the lack of a phenotype in transgenic mice that overexpress LTGF-β in mammary gland [11•]. A recent study in TGF-β-null heterozygotes, however, has shown there is a dramatic loss of circulating and tissue TGF-β levels as a result of the

Figure 2



Transforming growth factor (TGF)-β production, secretion, and activation. Some elements of protein processing and post-translation modifications involved in the regulatory control of TGF-β are depicted. The TGF-β_1 gene encodes a 390 amino acid polypeptide that is cleaved into two polypeptides that form homodimers during protein processing: latency-associated peptide (LAP) and TGF-β. These homodimers are noncovalently associated to form the small latent TGF-β complex, which is secreted. Alternatively, this complex can be covalently linked by disulfide bonds to a latent TGF-β binding protein (LTBP) before secretion. LTBP provides means of anchoring latent TGF (LTGF)-β in the extracellular matrix (ECM), which may involve cross-linking by transglutaminase and which requires proteolytic processing to release LTGF-β before activation. Activation occurs extracellularly to release TGF-β at or near the cell surface so that it immediately binds to its receptors. TGF-β receptors I and II form a heterocomplex that signals via the SMAD signal transduction protein family. TGF-β receptor III, also known as betaglycan, is nonsignaling but may be involved in presenting TGF-β to its signaling receptors.

haploid genotype [12•]. This surprising finding indicates that TGF-β plays a significant role in its own regulation.

A variety of immunostaining studies have been conducted in both murine and human tissues. These are difficult to summarize because of differences in tissue preparation, the antibodies employed, and their uncertain relevance to understanding the functional role of TGF-β *in situ*. Several studies localized TGF-β to the human breast epithelium and stroma [13,14], but at least one study [15] found it to be associated only with epithelial cells; this is probably due to either the antibody or fixative (see below). TGF-β is also found in the conditioned media of cultured normal human epithelial cells [16–18] and fibroblasts [18,19]. A recent study that immunolocalized TGF-β receptors I, II, and III in normal human breast [20] demonstrated that these are widely expressed in the breast epithelium and stroma.

Robinson *et al* [21•] used Northern blot analyses, *in situ* hybridization, and immunocytochemistry to define TGF-β expression patterns during murine mammary gland development and differentiation to establish that all three mammalian TGF-β isoforms are expressed in the epithelium during all phases of mammary development. TGF-β_2 was less abundant, however, whereas TGF-β_3 was the only isoform found in myoepithelial cells. TGF-β_1 transcripts and immunoreactivity decreased during pregnancy, but those of TGF-β_2 and TGF-β_3 increased until the onset of lactation [210•]. Intraepithelial immunoreactivity in that study corresponded to transcript localization, indicating sites of synthesis. Immunolocalization using antibodies to mature TGF-β versus TGF-β_1 LAP showed an interesting pattern of deposition that was suggested to indicate that TGF-β activity inhibits proliferation and its depletion is necessary for growth [22]. Although activation was implicit in the interpretation of the immunostaining, the model did not provide confirmation of activity, due in large part to the lack of reagents to differentially localize active TGF-β versus LTGF-β.

Studies in our laboratory demonstrate that under certain conditions some antibodies can discriminate between active and latent TGF-β [23]. We determined the specificity of TGF-β antibodies by using a immunodetection method that preserves endogenous LTGF-β in conjunction with engineered control tissues that specifically produce latent versus constituitively active TGF-β [24••]. Our extensive screening of available TGF-β antibodies demonstrated that antibody source, fixation, and tissue preparation influences the ability to discriminate between active and latent forms [25•]; thus, retrospective interpretation of other immunostaining protocols and antibodies combinations is not possible.

One of the most striking features of our immunolocalization in mammary gland using antibodies that discriminate between TGF-β and LTGF-β is the contrast between the highly restricted localization of TGF-β compared with the broad distribution of LTGF-β. Our initial studies concerned the effect of ionizing radiation on the mammary gland microenvironment. We observed that the immunoreactivity of activation-specific antibodies LC(1-30) and CC(1-30) is restricted to the normal mammary epithelium [26], whereas LAP immunoreactivity is widely distributed in the epithelium, fibrous stroma, and adipose stroma [24••]. A remarkably rapid (within 1 h) shift from predominantly LAP to TGF-β immunoreactivity in all of these tissue compartments led us to conclude that ionizing radiation leads to LTGF-β activation [24••]. Our conclusion is supported by additional studies that demonstrated that radiation induces stromal extracellular matrix (ECM) remodeling (eg collagen III), a known target of TGF-β action [26], and that administration of TGF-β-neutralizing antibodies blocks ECM remodeling in irradiated animals [27]. These studies indicated that, despite the apparent abundance of LTGF-β in normal, nulliparous mammary gland, active TGF-β is highly localized.

Transforming growth factor-β responses in cultured cell models

Much of our understanding of the biology of TGF-β comes from studies of cultured cells exposed to recombinant or purified active TGF-β. Nonetheless, the specific physiologic roles of the action of TGF-β in mammary gland are not well understood, which is due in part to the complexity of its effects on different cell types that is compounded by regulation at multiple levels and modified by cellular context [23]. Examples of these effects are summarized below. TGF-β has been shown to inhibit profoundly proliferation of normal rodent and human mammary epithelial cells in cell culture, and may also modify the production of differentiated proteins (for review [28]). Both cell type and context influence TGF-β action. 3T3 cell preadipocytes spontaneously activate their own secreted LTGF-β, whereas mature adipocytes do not, which implicates TGF-β activation as a potent negative regulator of adipocyte differentiation [29]. Fetal versus adult fibroblasts [30], young versus old smooth muscle cells [31], and normal human mammary epithelial cells grown on ECM versus plastic [32] are differentially sensitive to TGF-β growth inhibition. In addition to reversible growth inhibition, TGF-β can also induce apoptosis. Hormone withdrawal in hormone-dependent tissues such as prostate [33•] and uterus [34•] is a potent inducer of TGF-β-mediated apoptosis. In mammary gland, TGF-β mRNA increases early in involution and its activity is thought to mediate this apoptotic remodeling process [35]. Notably, mammary secretions during involution contain high levels of active TGF-β and extremely high levels of LTGF-β that regulate the mononuclear phagocytes involved in apoptotic remodeling of the gland [36].

TGF-β-induced ECM contributes to growth control in cultured cells [37] and may be involved in the changes in ECM during mammary gland development [13,22] and the menstrual cycle [38]. The cessation of DNA synthesis and end-bud regression caused by exogenous TGF-β-induced ECM is postulated to involve the concomitant induction of periepithelial ECM around end-buds [39]. This regulation may be offset by feedback mechanisms as evidenced by suppression of TGF-β-induced ECM mRNA levels in murine mammary cells cultured within a basement membrane-type ECM [40]. Even cells that lack a proliferative response to TGF-β-induced ECM, such as breast cancer cells, may still respond with specialized ECM production [16], that in turn may modify the tissue microenvironment in such a way as to affect growth. For example, recent mammary cell culture studies [41] also point to a role of ECM in modulating hormone response. The antiestrogen tamoxifen affects stromal production and activity of TGF-β, which has been postulated to contribute to its therapeutic benefit [42]. An interesting model of hormone-mediated remodeling in uterine tissue indicates that stromal activation of TGF-β mediates epithelial production of the proteases that are necessary for degradation of basement membrane [43•].

The addition of TGF-β to mammary cell cultures supports a role in regulation of morphogenesis and control of differentiation. TGF-β suppressed the ability of mammary explants cultured with lactogenic hormones to secrete casein [44]. It abolished ductal morphogenesis by human mammary epithelial cells cultured within a basement membrane-type ECM, whereas antibodies to TGF-β stimulated duct formation [45]. In another study [46], however, cultured mammary cell lines exhibited a biphasic response to TGF-β concentration; picomolar concentrations inhibited branching morphogenesis, whereas fentomolar concentrations stimulated it. Furthermore, chronic TGF-β exposure can elicit phenotypic transformation under certain conditions, resulting in the acquisition of mesenchymal-like transformation of mammary epithelial cells [47], or myofibroblast characteristics in stromal cells [48]. The role of such phenomena *in vivo* remains obscure, but may contribute to desmoplasia and invasive behavior in neoplasia.

Manipulating transforming growth factor-β activity in the mammary gland

The consequences of endogenous TGF-β activity in the mammary gland have been evaluated by using exogenous delivery of TGF-β or neutralizing antibodies. The pivotal observations by Daniel and coworkers [4••,5], who administered exogenous TGF-β via diffusion from miniature inorganic pellets, showed that end-buds undergo reversible regression during puberty, whereas alveolar buds in pregnancy, which are also actively proliferating, do not. During puberty, end-bud DNA synthesis was profoundly inhibited within 12 h of exposure to TGF-β-containing implants, and by 48 h this resulted in an 80–90% decrease in end-bud number [4••]. In contrast, DNA synthesis in distal ductal epithelium and fibroblasts was unaffected, indicating an underlying difference in responses and/or access to these cells. End-bud regression was preceded by the deposition of a thick, collagen- and glycosoaminoglycan-rich ECM, which was postulated to force premature differentiation [49]. This selective regression of end-buds was reversible if the implant was removed, even after long-term exposures [5], indicating that the gland did not become refractory or lose its multipotent stem-cell population. In contrast, exogenous TGF-β did not inhibit lobuloalveolar growth, morphogenesis or differentiation. This stage specificity belies a simple role for TGF-β as a DNA synthesis inhibitor and points to the possible differences in how these two actively proliferating structures are regulated. It may also indicate that endogenous TGF-β activity is differentially regulated during these distinct phases of concomitant mammary gland growth and differentiation.

Transgenic manipulation of TGF-β in mammary gland underscores the importance of defining when and where

the protein is active. As mentioned above, overexpression of LTGF-β does not produce a mammary phenotype. TGF-β can be produced in a constitutively active form by expression of a construct in which the cysteines at positions 223 and 225 are mutated to serines, however, preventing dimerization of LAP [50]. Two transgenic models have been created with dramatically different phenotypes, depending on the promoter that is used to drive expression of the transgene. Murine mammary tumor virus (MMTV)-long terminal repeat promoter is used to direct transgene expression to the mammary epithelium. When TGF-$\beta^{223\text{-}225}$ is expressed on an MMTV promoter, the gland is transiently hypoplastic during ductal morphogenesis, but recovers and is able to lactate and support offspring without apparent difficulty [11•]. It has been suggested by Smith [51] that additional expression of the MMTV-driven transgene in the salivary gland may influence this phenotype. If the mutated constitutively active TGF-β is driven by the whey acidic protein (WAP) promoter, however, a milk protein that is highly expressed during pregnancy and lactation, ductal morphogenesis is unaffected, but alveolar development is greatly compromised [52••].

Subsequent experiments demonstrated that proliferation was uninhibited but that apoptosis was stimulated during both estrous and pregnancy [53••]. Furthermore, transplant experiments indicated that the action of TGF-β was intrinsic to the epithelial cells and acted in an autocrine manner. For example, wild-type epithelium was unaffected and differentiated normally [53••]. Taking full advantage of the mammary model, Kordon *et al* [53••] used serial transplantation of WAP-TGF-$\beta^{223\text{-}225}$ epithelium to show that the mammary stem cells were significantly compromised by the transgene expression. Because endogenous WAP protein or reporter expression is restricted to specific luminal epithelial cells and is regulated by the estrus cycle, those investigators postulated that WAP-TGF-$\beta^{223\text{-}225}$ expression targeted the immediate daughters of mammary epithelial cells, causing inappropriate apoptosis and subsequent depletion of this compartment. The severity of the MMTV-TGF-$\beta^{223\text{-}225}$ phenotype may also be influenced by the timing or cell specificity of expression relative to that of endogenous LTGF-β activation. Our preliminary data using antibodies that discriminate between active and latent TGF-β supports this concept in that TGF-β activation is suppressed by mid to late pregnancy. Thus, WAP-TGF-$\beta^{223\text{-}225}$ would be driving inappropriate TGF-β activity at a point when it is normally inhibited.

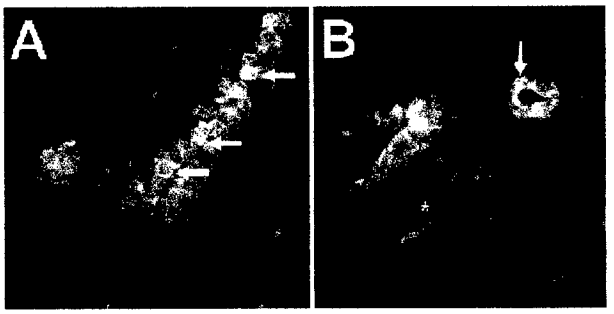
The question of how mammary glands of TGF-β-null mice develop has not yet been addressed because the null mice die around the time of mammary gland development [54]. These mice have provided evidence of a previously unrecognized aspect of TGF-β biology, however. Letterio *et al* [55] demonstrated that homozygous TGF-β-null offspring are protected from the gross inflammation that leads to their early death by maternal transfer of TGF-β via the placenta and milk. Another surprising aspect of the TGF-β_1-null mouse is that the haploid genotype leads to greatly reduced TGF-β production, which is reflected by serum levels that are 11% of the levels in wild-type animals, indicating that TGF-β regulates its own production and/or stability. This chronic depletion of the latent complex pools presumably restricts TGF-β activity, and results in a subtly altered proliferative phenotype in liver and lung. A further consequence is increased tumor development, indicating that TGF-β is sufficiently depleted to impede its action as a tumor suppressor [12•].

We recently evaluated the mammary gland phenotype of TGF-β-null heterozygote mutant mice (Barcellos-Hoff MH, *et al*, unpublished data). Mammary ductal outgrowth during puberty was accelerated, and mammary epithelial proliferation was increased in young TGF-β_1 haploid genotype animals. This effect appeared to be stage-specific in that mammary gland whole mounts from adult nulliparous mice were grossly normal in morphology and functionally intact. Despite the apparently normal morphology, we found that the frequency of proliferating cells of the mammary epithelium is significantly elevated in mammary gland, as occurs in other tissues like liver from TGF-β-null mutant heterozygotes [12•].

It is unknown at this time whether systemic, stromal, epithelial or all sources of TGF-β are critical in defining the rate of growth in mammary gland. Gorska *et al* [56] addressed this question by overexpressing a dominant-negative kinase-deficient TGF-β type II receptor driven by MMTV-long terminal repeat, resulting in primarily epithelial expression, or metallothionein-derived promoter, which is expressed in the stroma. The dominant-negative receptor impedes signaling by all TGF-β isoforms, but circumvents the issues raised by constitutively active TGF-β transgenes. Blunting the response to TGF-β in the mammary epithelium resulted in alveolar hyperplasia and premature functional differentiation. In contrast, inhibition of the stromal response to TGF-β by this means resulted in increased ductal branching [57]. Thus, both stromal and epithelial responses to TGF-β activity play a role in defining the maturation of mammary tissue.

The question of when and where LTGF-β is activated during normal development and homeostasis remains to be addressed, however. We recently developed dual immunofluorescence of LAP antibodies and activation-specific chNTGF-β antibodies [27] and have begun to study the pattern of localization during mouse mammary gland development and differentiation (Fig. 3). Although LAP immunoreactivity (green) is relatively uniform, a heterogeneous pattern of TGF-β staining (yellow-orange) is observed in the epithelium of adult mammary glands (Barcellos-Hoff MH *et al*, unpublished data). We have exam-

Figure 3



Dual immunolocalization of latency-associated peptide (LAP) and transforming growth factor (TGF)-β in murine mammary gland. Antibodies to LAP (green) to localize latent TGF (LTGF)-β, antigen-purified TGF-β antibodies that specifically detect active TGF-β (red), and DAPI stained nuclei (blue) were visualized using tricolor digital fluorescence microscopy. **(A)** A section tangential to a duct; **(B)** a transverse and cross-section of a duct of mammary gland from a nulliparous Balb/c mouse. Colocalization of LAP and TGF-β appears yellow in certain epithelial cells (arrows). Note that certain cells have prominent LAP staining, but are not immunoreactive for TGF-β and are adjacent to cells that exhibit staining of both LAP and TGF-β. Neither stromal, myoepithelial, or endothelial (B, asterisk) cells show prominent TGF-β immunoreactivity.

ined mammary glands from a variety of developmental states to determine the physiologic correlates of this pattern. We found that mammary epithelial TGF-β immunoreactivity was heterogeneous and most intense during periods of proliferation and morphogenesis, suggesting the presence of a distinct subpopulation. Further characterization of these cells and determination of their ultimate fate in terms of proliferation and differentiation may be informative regarding the cellular mechanisms by which pattern and function are established during mammary gland development.

Conclusion

It is clear from a variety of studies in cell culture that TGF-β can have myriad effects, many of which complement each other, but others that appear paradoxic. *In vivo* studies using exposure to exogenous sources or transgenic expression of constitutively active protein have shown what TGF-β can do in the mammary gland. These have generally led to the conclusion that TGF-β has a prominent role in regulating pattern formation by the epithelium, perhaps via interactions with the stroma, and is involved in fate decisions by individual cells. In the great majority of animal and cell culture studies, however, activation of the ubiquitous latent TGF-β complex, which exercises fundamental control of TGF-β action, has been scarcely addressed, which is due in large part to the lack of appropriate tools and reagents. As evidenced by the WAP-TGF-$\beta^{223-225}$ versus the MMTV-TGF-$\beta^{223-225}$ transgenic phenotypes, spatial and temporal patterns of LTGF-β activation are key elements for to understanding the specific consequences of TGF-β activity during mammary gland development. Likewise, similar studies of the relative activity of the different TGF-β isoforms in the mammary gland may help shed light on the need for this apparent redundancy. Finally, studies that address mechanisms of activation may then contribute to strategies for manipulation of TGF-β *in situ* and lead to a better understanding of how its dysregulation contributes to carcinogenesis [58].

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Appendix II

[CANCER RESEARCH 60, 1254–1260, March 1, 2000]

Irradiated Mammary Gland Stroma Promotes the Expression of Tumorigenic Potential by Unirradiated Epithelial Cells[1]

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ABSTRACT

We have shown that ionizing radiation, a known carcinogen of human breast, elicits rapid, persistent, and global changes in the mammary microenvironment as evidenced by altered extracellular matrix composition and growth factor activities. To address whether these events contribute to radiogenic carcinogenesis, we evaluated the effect of irradiated mammary stroma on the neoplastic potential of COMMA-D mammary epithelial cells. Although COMMA-D cells harbor mutations in both alleles of p53, they are nontumorigenic when injected s.c. into syngeneic hosts. Unirradiated COMMA-D cells transplanted to mammary fat pads cleared previously of epithelia preferentially formed tumors in irradiated hosts. Tumor incidence at 6 weeks was 81% ± 12 SE when animals were irradiated with 4 Gy, 3 days prior to transplantation, compared with 19% ± 2 SE ($P < 0.005$) in sham-irradiated hosts. This effect was evident when cells were transplanted 1 to 14 days after irradiation. Furthermore, tumors were significantly larger (243.1 ± 61.3 mm^3 *versus* 30.8 ± 8.7 mm^3) and arose more quickly (100% by 6 weeks *versus* 39% over 10 weeks in sham hosts) in fat pads in irradiated hosts. The contribution of local *versus* systemic effects was evaluated using hemibody (left *versus* right) irradiation; tumors formed only in fat pads on the irradiated side. These data indicate that radiation-induced changes in the stromal microenvironment can contribute to neoplastic progression *in vivo*. Disruption of solid tissue interactions is a heretofore unrecognized activity of ionizing radiation as a carcinogen.

INTRODUCTION

The multistep model of carcinogenesis defines cancer initiation as genomic change and promotion as the series of events leading to proliferation of initiated cells. Agents that promote carcinogenesis are generally thought to increase the probability that a cell will acquire additional mutations necessary for neoplastic progression. Quantitative studies in rodent models demonstrated that the number of cells initiated, as evidenced by morphological transformation in culture after either physical or chemical carcinogen exposure, far exceeds the number of tumors that develop *in vivo* (1, 2). Because initiation appears to be a frequent event (3), the factors that facilitate expression of tumorigenic potential are critical in determining cancer frequency but are still poorly understood.

The contribution of stroma to early events in carcinogenesis has recently begun to be appreciated. Whereas it is well-recognized that tumors can recruit cells (*e.g.*, endothelial cells) and induce changes in the stroma that are conducive to their growth (4, 5), it has also been suggested that disruption of stromal/epithelial interactions may provide a stimulus for initiated cells to move further down the neoplastic pathway (6, 7). Specialized microenvironments, composed of insoluble ECM[3] and soluble growth factors, mediate epithelial-stromal interactions and play a pivotal role in normal tissue development and function (8, 9). Such interactions can efficiently suppress the expression of the neoplastic phenotype (3, 10, 11). Conversely, abnormal stromal/epithelial interactions have been shown to enhance the ability of cells to express the neoplastic phenotype (12, 13). Transgenic manipulation of the microenvironment, rather than the target cell, can also stimulate tumorigenesis (14). Recent studies in chronic myelogenous leukemia and in human mammary cancer cells indicate that reestablishing appropriate interactions with the ECM can reverse tumorigenesis and neoplastic behavior, even in the presence of grossly abnormal genetic damage (15, 16).

Ionizing radiation is a complete carcinogen, able both to initiate and promote neoplastic progression (17), and is a known carcinogen of human breast (18–21) and rodent mammary glands (22–24). Our previous studies have demonstrated that radiation exposure elicits rapid and persistent global remodeling of the mammary gland ECM (25). Because cell-ECM interactions are pivotal in mammary differentiation and growth control (26, 27), we postulated that the radiation-induced mammary microenvironment may contribute to radiation carcinogenesis by disrupting cell interactions in a manner that is conducive to expression of neoplastic potential (28).

To test the above hypothesis, we transplanted epithelial-free mammary stroma with the functionally normal COMMA-D mammary epithelial cell line. The COMMA-D mammary epithelial cell line arose spontaneously from a primary culture of epithelial cells from mammary glands of pregnant BALB/c mice and is nontumorigenic at early passages (29). It was shown subsequently to contain two mutations in p53 characterized as substitution of Trp for Cys at codon 138 and deletion of the first 21 nucleotides of exon 5 (30), which result in nuclear accumulation (31). The mutations in p53 in these cells suggest that the population may be genomically unstable and susceptible to neoplastic transformation (30).

We show here that COMMA-D cells transplanted to cleared mammary glands in irradiated hosts gave rise to significantly more tumors that arose more quickly and grew larger than in unirradiated animals. Hemibody irradiation supports the conclusion that radiation effects on mammary microenvironment is critical because tumors arose only when COMMA-D cells were transplanted to the irradiated side of animals. These data demonstrate that radiation effects on stroma facilitate expression of neoplastic potential in the absence of exogenously induced mutagenic events in mammary epithelial cells.

MATERIALS AND METHODS

Animals. Female BALB/c mice were obtained from Simonson (Gilroy, CA) and housed five per cage with chow and water *ad libitum* in a temperature- and light-controlled facility. When necessary, mice were anesthetized using xyalzine (40 mg/kg) and ketamine (25 mg/kg) injected i.m. Carbon dioxide inhalation was used to kill the animals in accordance with Association for Assessment and Accreditation of Laboratory Animal Care guidelines and institutional review and approval. The fourth inguinal mammary glands were removed for histology and whole mounts.

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[3] The abbreviations used are: ECM, extracellular matrix; CFP, cleared fat pad; TGF-β, transforming growth factor-β1; DAPI, 4′,6-diamidino-2-phenylindole.

Cell Culture. COMMA-D mouse mammary epithelial cells originating from midpregnant BALB/c mice (29) were obtained at passage 11 from Dr. Peggy Neville (University of Colorado, Denver, CO) and were expanded and frozen at passage 13. Cells were grown routinely in 1:1 F12:DMEM supplemented with 2% fetal bovine serum, epidermal growth factor (5 ng/ml), insulin, transferrin, and selenium on tissue culture plastic in a 5% CO_2 humidified incubator at 37°C and passaged twice weekly at <90% confluency. Cells were used between passages 15 and 24. Differential trypsinization was used to enrich for morphological variants at passage 22 by collecting cells released in the first 3 min of trypsinization (CD-3T), adding trypsin for an additional 2 min (discarded), followed by an additional 5 min of trypsinization to release the remaining cells (CD-5R). Each subpopulation was expanded one passage, and aliquots were frozen for subsequent characterization. Cells were trypsinized, counted, and resuspended in serum-free medium or PBS immediately before transplantation.

Transplantation Assay. The female mammary gland is unique among all glands in that the epithelium develops postnatally from a rudiment that can be removed from the inguinal glands at ~3 weeks of age (32). Surgical removal of the parenchyma results in a gland-free mammary fat pad, referred to as a CFP, suitable for receiving donor tissue at the time of clearing or later (32). Transplantation of normal mammary epithelial cells produces ductal outgrowths that fill the fat pad and are nearly indistinguishable in whole mounts or histologically from intact gland (32). Anesthetized 3-week-old mice were opened s.c. at the anterior midline and along each flank to reveal the inguinal mammary gland. The epithelium was destroyed by cauterizing the connection to the 5th gland, and removing the tissue from the nipple to just above the lymph node, leaving approximately two-thirds of the fat pad. The procedure was repeated on the opposite side, and the skin was closed with wound clips.

Mammary glands were cleared of epithelia at 3 weeks of age. The mice were irradiated and transplanted at 10–12 weeks of age. The CFPs were exposed from the rostral midline to avoid the scar tissue formed by clearing. Known numbers of COMMA-D cells were injected using a Hamilton syringe into the fat pad in a volume of 10 μl. Animals were monitored weekly for gross changes and sacrificed at 6–10 weeks as indicated, and the inguinal glands were removed for analysis. Glands were fixed in 100% ethanol:glacial acetic acid (3:1) for 24 h, followed by alum carmine, according to published methods (33). Tumor sizes were determined by caliper measurements of width, thickness, and length in the whole mounts, and the approximate tumor volume was calculated as the product of these. Selected tissues were fresh frozen in OCT (Miles Associates, St. Louis, MO).

Irradiation. Unanesthetized mice were whole-body irradiated in perforated Plexiglas tubes using ^{60}Co γ-radiation at a dose rate of 0.32 Gy/min to a total dose of 4 Gy. Dosimetry was determined using an Victoreen ionization chamber. Anesthetized animals were used for hemibody irradiation (4 Gy) by shielding the right lateral half with lead collimators. Control animals were sham irradiated with or without anesthesia, as indicated.

Immunofluorescence. Cells were seeded in 24-well plates in 1 ml of 0.5% serum-containing medium. After reaching confluence, cells were washed with PBS and fixed with 500 μl of methanol for 10 min at −20°C for vimentin and keratin staining and in methanol overnight at 4°C for p53 immunostaining. Fixed cells were stored in PBS at 4°C before immunostaining. Cryosections (5 μm) were fixed with 2% buffered paraformaldehyde for 20 min for p53 immunostaining and 4% buffered paraformaldehyde for 10 min for vimentin and keratin immunostaining.

Polyclonal rabbit antibodies against p53 protein (CM5; Vector Laboratories, Burlingame, CA) were used at 1:500 (0.5 μg/ml). Monoclonal mouse antibodies against vimentin (clone VIM 13.2; Sigma) were used at 1:200 dilution (10 μg/ml), and polyclonal guinea pig antibodies to keratin (Sigma) were used at 1:100 dilution.

Nonspecific reactivity was blocked with the supernatant of 0.5% casein stirred for 1 h in PBS. Specimens were incubated with 50 μl of primary antibody overnight (18–20 h) at 4°C. Vimentin and pan-keratin primary antibodies were incubated together, followed by sequential incubation with fluorochrome-labeled secondary antibodies for 1 h at room temperature. Nuclei were counterstained with DAPI at 0.5 μg/ml (Sigma) during the last wash. Specimens were mounted with Vectashield mounting medium (Vector Laboratories) and stored at −20°C prior to viewing. Whole mounts were embedded in paraffin and sectioned at 5 μm for standard histology staining with H&E. Sections from whole mounts were incubated with p53 antibodies as above and detected with alkaline phosphatase-linked secondary antibody.

Image Acquisition and Processing. Fluorescence microscopy of double or triple fluorescence-stained sections were imaged using a Zeiss Axiovert equipped with epifluorescence and multiband pass filter and differential wavelength filter wheel. Images were acquired using a scientific-grade 12-bit charged coupled device (KAF-1400, 1317 × 1035 6.8-μm square pixels) camera (Xillix, Vancouver, Canada). The images were captured so that intensities for a given experiment fell within the 12-bit linear range. Relative intensity was maintained when constructing figures by scaling the data set to a common 8-bit scale using Scilimage (TNO, Delft, the Netherlands).

Statistical Analysis. Tumor incidence was evaluated using 95% confidence intervals and the z test of proportion for data derived from a single experiment or two-tailed t test for data from multiple experiments (SigmaStat, Santa Rosa, CA). The significance of differences between tumor size for sham-irradiated control and irradiated animals were determined using two-tailed Mann-Whitney rank sum test (34).

RESULTS

COMMA-D Cells Retain Mammary Developmental Potential and Are Nontumorigenic. COMMA-D cells are functionally intact in that they can be induced by lactogenic hormones to synthesize a number of milk proteins, including β-casein (35). We confirmed that the cell line also retains the capacity to produce ductal outgrowths *in vivo* when early-passage cells are transplanted to CFPs (29) and undergo morphogenic reorganization into acini when cultured on a complex basement membrane-type matrix (25). When transplanted into CFPs of 3-week-old mice, COMMA-D cells form a simple mammary ductal outgrowth that fills 25–100% of the stroma. These outgrowths were confirmed as originating from COMMA-D cells by nuclear p53 immunoreactivity, which was absent from stromal cells (not shown). Tumors were not observed when COMMA-D cells were transplanted at the time of clearing in 3-week-old animals or when injected s.c. into adult syngeneic hosts (0.5, 1, or 2 million) over a period of 8 weeks (n = 6, not shown).

COMMA-D Cells Transplanted to CFPs of Irradiated Hosts Establish Tumors Rapidly. Our previous studies indicated that ionizing radiation leads to global remodeling of the ECM and induces activity of potent modulators of epithelial behavior (25, 36, 37). Individually irradiated mammary glands exhibit the same microenvironment changes as those from whole-body-irradiated animals, indicating that these effects are mediated by local factors.[4] CFPs from irradiated mice show similar remodeling of the microenvironment as that observed in intact mammary glands (not shown).

We tested whether the irradiated mammary microenvironment could modulate the neoplastic potential of COMMA-D mammary epithelial cells. Different numbers of unirradiated COMMA-D cells were transplanted to CFPs in adult mice that were sham-irradiated or that received 4 Gy ^{60}Co γ-radiation 3 days prior to transplantation (Fig. 1). A single tumor formed in sham-irradiated CFPs transplanted with 2×10^6 cells (n = 6); no tumors were observed when fewer cells were injected. However, tumors arose in irradiated CFPs as a function of cell number, even when transplanted with as few as 2.5×10^5 cells. Every CFP (100%) from irradiated hosts contained tumors when injected with 2×10^6 unirradiated COMMA-D cells. In four independent experiments (n = 36 injected CFPs), tumor incidence was significantly ($P < 0.005$, two-tailed t test) greater in irradiated CFPs (30 of 36) *versus* sham-irradiated (8 of 36). The percentage of CFP-bearing tumors averaged 19% ± 2 SE in sham-irradiated hosts and 81% ± 12 SE in hosts irradiated with 4 Gy, 3 days prior to transplantation.

[4] E. J. Ehrhart and M. H. Barcellos-Hoff, unpublished data.

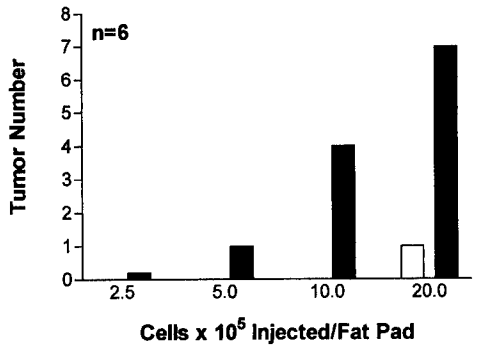

Fig. 1. Tumor incidence of COMMA-D cells as a function of cell number in sham-irradiated hosts (□) or total body irradiated hosts (■). Animals were irradiated with 4 Gy, 3 days before transplantation of cells to CFPs. The incidence of tumors was significantly ($P < 0.05$) greater in irradiated hosts transplanted with 10^6 cells or more.

Ductal outgrowths were obtained at low frequency (~25%) in adult mice, but the frequency and character were similar in irradiated and sham-irradiated CFPs. In addition, we found that the transplantation take of normal mammary tissue fragments to sham and irradiated hosts was equivalent.[5] Thus, the ability of cells to survive and/or grow in mammary glands in both sham and irradiated hosts was similar. Some tumors were found in association with otherwise normal-appearing ductal outgrowths (Fig. 2*A*), but most were isolated.

Nuclear accumulation of p53 was evident in ductal outgrowths and tumors and was lacking from cells in adjacent stroma (Fig. 2*B*). Tumors were both vimentin and keratin positive and exhibited nuclear p53, indicative of their origin from COMMA-D cells (Fig. 2*C*). Note that p53 immunostaining was heterogeneous but more evident using the more sensitive immunofluorescence detection. This heterogeneity is also evident in culture (Fig. 4).

The persistence of radiation effects was determined by transplanting COMMA-D cells to CFPs as a function of time after exposure to 4 Gy whole-body ^{60}Co γ-radiation exposure (Fig. 3). All irradiated animals showed a significant ($P < 0.05$) increase in tumor incidence, ranging from 100% at 3 day to 58% at 14 days after irradiation compared with 25% in the sham-irradiated host. The peak occurred when irradiated animals were transplanted at 3 days after irradiation, at which time tumors arose in 100% of glands in irradiated hosts. Furthermore, the mean size of tumors from irradiated animals was 243 $mm^3 \pm 61$ SD compared with 31 $mm^3 \pm 9$ SD in the few tumors that arose in sham-irradiated hosts. The mean size of tumors in irradiated animals at all times after irradiation were significantly ($P < 0.05$; Mann-Whitney rank sum test) larger than the size of tumors in sham-irradiated control animals.

To test whether tumor formation was accelerated in the irradiated CFPs, we evaluated tumor incidence as a function of time after transplantation. Tumors arose too quickly in irradiated mammary glands (100% by 6 weeks) to permit extending their observation period. The overall tumor incidence in sham-irradiated hosts over a period of 10 weeks was 39% of transplanted CFPs examined. Tumor incidence decreased from 6 to 10 weeks in sham-irradiated hosts, suggesting that these small tumors may regress. Thus, had we been able to carry the irradiated mice to 10 weeks, the difference between the two groups would be even more dramatic.

COMMA-D Parental Heterogeneity Gives Rise to Subpopulations That Are Also Preferentially Tumorigenic in Irradiated Hosts. Clonal COMMA-D subpopulations have been shown to retain the capacity to produce ductal outgrowths and to respond to hormone stimulation (38), suggesting that some COMMA-D cells have multipotent characteristics of putative stem cells. The multipotent nature of these cells is also suggested by the observation that COMMA-D cell cultures contain multiple morphologically distinct cells consisting of flat, polygonal cells that form contact-inhibited monolayer islands and spindle-shaped cells that form ridges that surround the islands. This

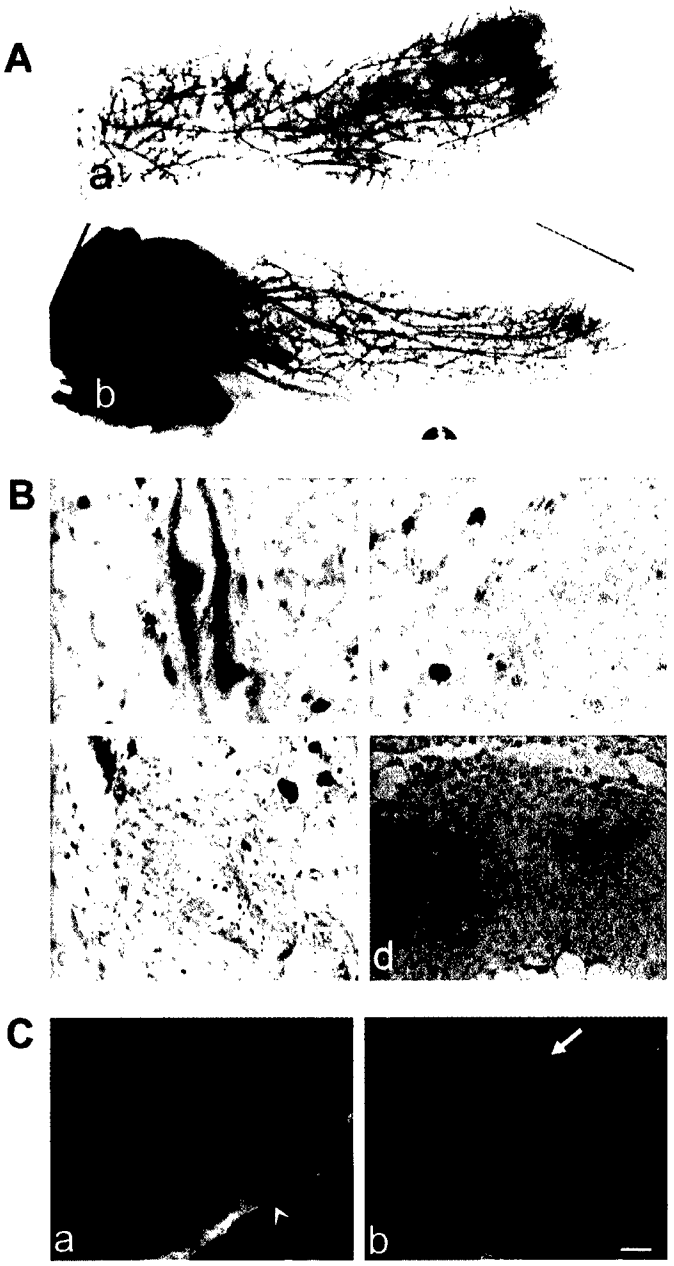

Fig. 2. *A*, whole mounts of COMMA-D outgrowths. COMMA-D cells were transplanted into CFPs at 10 weeks of age into sham (*a*) or irradiated (*b*) animals. Tumors, such as the one to the *left* of the image in *b*, were sometimes associated with otherwise normal-appearing ductal outgrowths. *B*, p53 immunoreactivity is found in ductal outgrowths and tumors. Enzymatic detection (*red*) of p53 by the CM5 antibody localized to nuclei in ductal outgrowths (*a*) *versus* tumors prepared from whole mounts of mammary glands that were embedded in paraffin for histology (*b*). Although p53 detection is compromised by the whole-mount preparation (compare frequency of positive cells between *Bb* and *Cb*), specific nuclear staining (*arrows*) is epithelial in outgrowths and heterogeneous in the tumor. Note lack of staining in the stromal cells around ducts or tumors. Controls in which the primary antibody was deleted for sections of outgrowths (*c*) and tumors (*d*) show diffuse, nonspecific background and black debris. *C*, characterization of tumors. *a*, dual immunofluorescence localization of vimentin (*green*) and keratin (*red*) demonstrate keratin staining of tumor cells. Nuclei are counterstained with DAPI (*blue*). *Arrowhead*, tumor capsule, which is vimentin-positive and keratin-negative. *b*, immunofluorescence localization of p53 (*green*) in tumor cryosections. Nuclei are counterstained with DAPI (*blue*). Note lack of p53 in stroma (*arrow*). *Bar*, 10 μm.

[5] Unpublished observations.

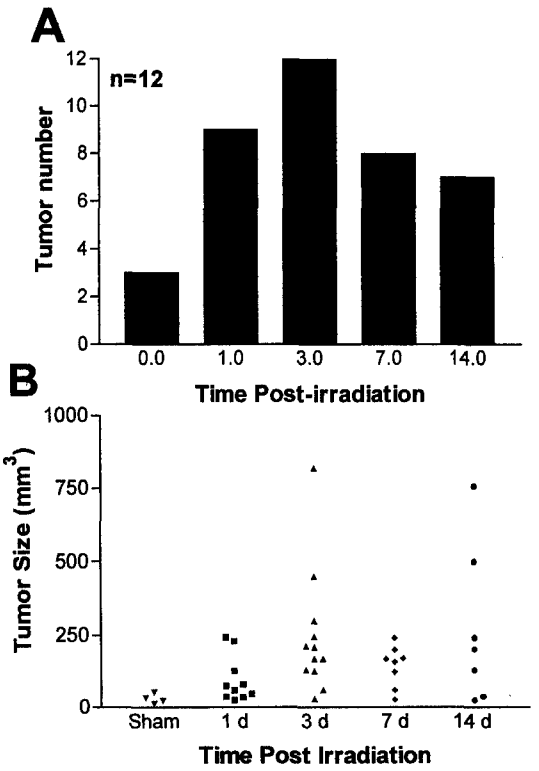

Fig. 3. Tumor frequency and size after transplantation of COMMA-D cells as a function of time after irradiation. *A*, tumor incidence ($n = 12$) was significantly ($P < 0.05$) greater in hosts irradiated 1, 3, and 7 days before transplantation. *B*, tumor size distributions. The median tumor sizes of the five treatment groups were significantly different ($P < 0.05$) based on Kruskal-Wallis ANOVA on ranks (34). *d*, days.

heterogeneity is maintained by routine passaging at 80–90% confluency. These cells are p53 positive and exhibit various levels of keratin and vimentin immunoreactivity (Fig. 4*A* and *E*). Keratin is observed predominantly in polygonal cells, whereas vimentin expression is less prominent and is associated with the ridges of spindle cells.

The possibility that these distinct cell types have different tumorigenic potentials or are preferentially selected during growth *in vivo* was addressed by testing their respective behaviors in CFPs. Morphologically distinct subpopulations were enriched from the parent population by differential trypsinization. Trypsinization releases spindle-shaped cells in the first 3 min (designated CD-3T), whereas predominantly polygonal cells remain after 5 min of trypsinization (designated CD-5R). Both cell types exhibited p53 immunoreactivity in culture (Fig. 4, *E–G*). The CD-3T subpopulation was primarily vimentin positive and keratin negative and had large nuclei (Fig. 4, *B* and *F*). The CD-5R subpopulation contained keratin-immunoreactive cells, with little expression of vimentin, and had small nuclei (Fig. 4, *C* and *G*).

Both the CD-3T and CD-5R subpopulations were significantly ($P < 0.05$) more tumorigenic in irradiated hosts when transplanted to sham- *versus* irradiated-hosts (Fig. 5). However, the CD-5R subpopulation produced fewer tumors (4 of 12) than the parent in irradiated CFPs and did not give rise to any tumors in the sham-host. The tumors from the CD-5R were also considerably smaller than either the parent or the CD-3T subpopulation (Fig. 5*B*). In contrast, the CD-3T subpopulation was more efficient (12 of 12) in generating tumors than the parent population (8 of 12) in this experiment. Furthermore, unlike the parent COMMA-D population, the size of tumors from the 3T-CD subpopulation were similar in the sham-irradiated CFPs and irradiated CFPs. Thus, the size of the tumors is influenced by both the nature of the microenvironment and of the epithelial population.

COMMA-D Cells Are Tumorigenic Only in the Irradiated CFPs of Hemibody-irradiated Mice. Radiation might promote neoplastic behavior by causing aberrant immune or endocrine function. Partial (left *versus* right) body ^{60}Co γ-irradiation of anesthetized mice was used to test whether systemic factors from the irradiated host contributed to tumor promotion in irradiated CFPs. No tumors were found in nonirradiated CFPs ($n = 8$), whereas three tumors were

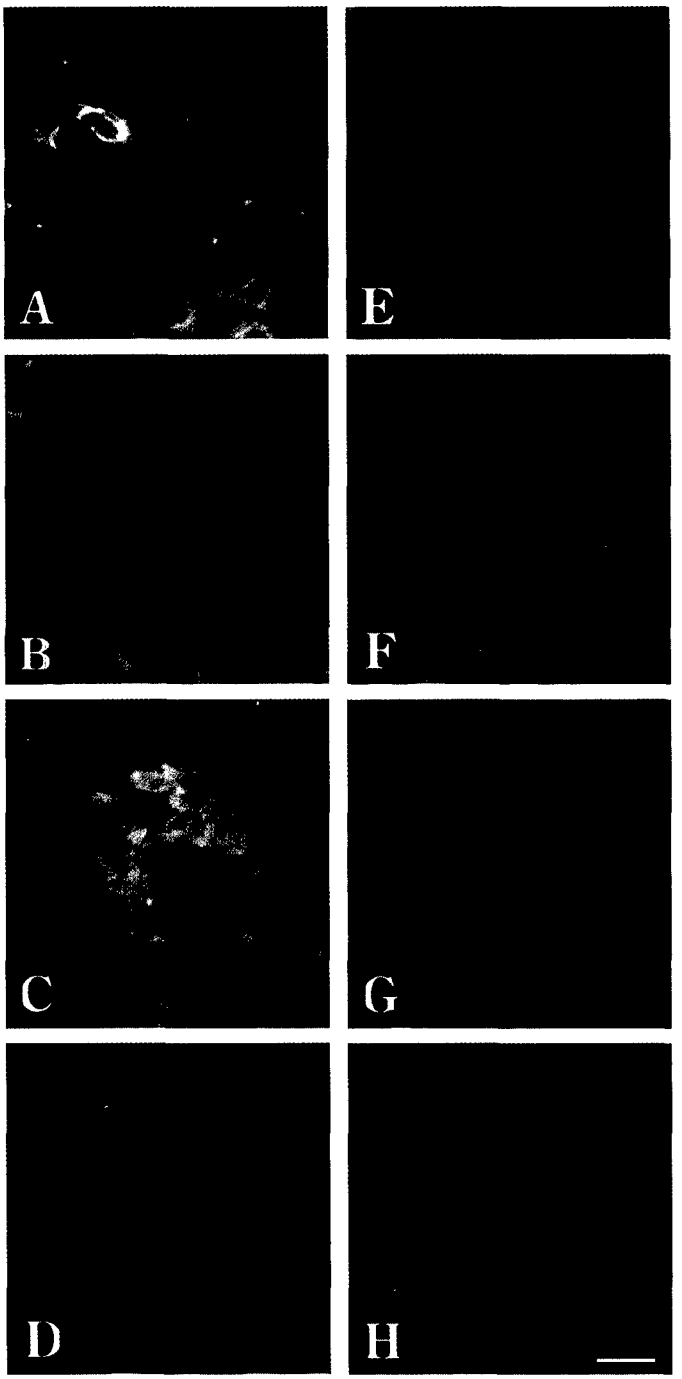

Fig. 4. Morphological and immunostaining characteristics of cultured COMMA-D mammary epithelial cells (*A* and *E*) and subpopulations, 3T (*B* and *F*) and 5R (*C* and *G*). Nuclei are counterstained with DAPI (*blue*). *A–D*, vimentin and keratin dual indirect immunofluorescence. The parent population contains cells that are vimentin positive (*green*), keratin positive (*red*), or both (*orange*). 3T cells are predominantly vimentin positive, whereas 5R contains cells that are both vimentin and keratin positive, as indicated by the *orange* color, indicative of fluorochrome colocalization. *E–H*, p53 immunoreactivity. Immunofluorescence localization of nuclear p53 (*green*) is heterogeneous in all three cell populations. *D* and *H*, antibody minus controls. Deletion of primary antibodies for vimentin and keratin (*left*) or p53 (*right*) is shown. *Bar*, 20 μm.

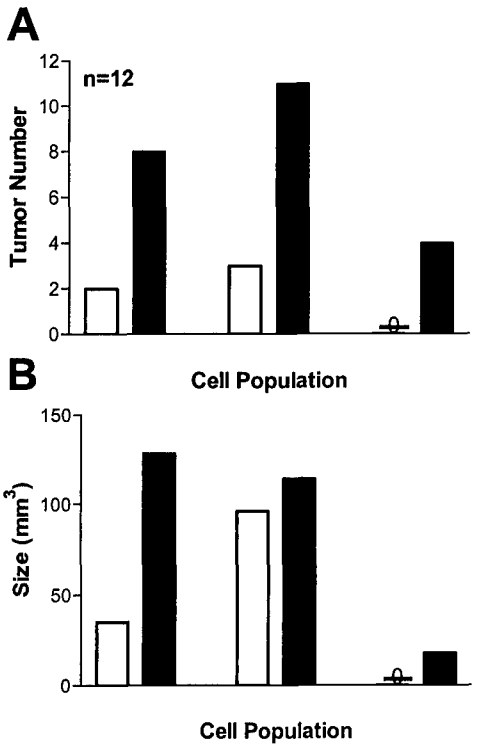

Fig. 5. Tumor formation by COMMA-D parent cells and subpopulations, CD-3T and CD-5R, transplanted into CFPs in sham-irradiated hosts (□) or hosts irradiated 3 days before transplantation with 4 Gy (■). *A*, tumor incidence was significantly ($P < 0.05$) greater in irradiated *versus* sham hosts for all three populations. *B*, tumor size varied as a function of cell population and of irradiation status.

observed in irradiated CFPs ($P = 0.05$). To determine whether the low incidence of tumor formation was attributable to the systemic effects of anesthesia necessary for hemibody irradiation, we asked whether anesthesia compromised tumor formation. Anesthetized, whole-body-irradiated mice formed tumors in two of eight transplanted CFPs, whereas unanesthetized, whole-body-irradiated mice in this experiment formed seven tumors in eight CFPs. Thus, the low incidence of tumors in the partial body protocol compared with whole-body exposures appears to be attributable to the use of general anesthesia; tumor incidence was restricted to CFPs on the irradiated side, indicating that local tissue effects were dominant over the systemic consequences of irradiation.

DISCUSSION

Although the stroma has generally been considered a silent bystander during epithelial carcinogenesis, the concept that the microenvironment is central to maintenance of cellular function and tissue integrity provides the rationale for the idea that its disruption can contribute to neoplasia (8). Indeed, it has been suggested that cancer may be a physiological response to an abnormal environment (6, 14, 39, 40), and evidence is accumulating that support such hypotheses (41). Most studies of radiogenic carcinogenesis emphasize radiation-induced DNA damage because mutations are believed to initiate carcinogenesis. However, low doses of ionizing radiation alter the composition of tissue microenvironment by rapidly affecting cytokine production and activities (36, 37), ECM composition (25, 37, 42), and expression of receptors that mediate cell-cell interactions (43–45).

The studies reported here address the question of whether radiation-induced changes in the composition of the mammary microenvironment promote the expression of neoplastic potential. We showed that COMMA-D mammary epithelial cells, which retain normal developmental capacity, were nontumorigenic by standard assays (lack of tumor formation when injected s.c. or in juvenile CFPs). Small, infrequent (19%) tumors were obtained when COMMA-D cells were transplanted to adult CFPs, suggesting that the physiological status of the host can modulate COMMA-D neoplastic potential. Tumorigenic efficiency increased to 100% when unirradiated COMMA-D cells were transplanted to CFPs in irradiated hosts. Furthermore, tumor size also substantially increased in irradiated CFPs. The effect of radiation on enhanced tumorigenesis persisted for at least 14 days after a single radiation exposure. We concluded that this was attributable to radiation-induced changes in the mammary stroma by comparing the irradiated and nonirradiated CFPs after hemibody irradiation; tumors formed only in the CFPs from the irradiated side. Thus, radiation exposure altered the mammary microenvironment in a manner that promotes the neoplastic behavior of these mammary epithelial cells, which harbor defective p53.

Although clonal, COMMA-D cells exhibit morphological and phenotypic diversity in culture (30, 38). We therefore determined some of the characteristics of the tumors arising from COMMA-D cells and found that tumors maintained cytokeratin immunoreactivity whether they arose in normal or irradiated CFPs. We then used selective trypsinization to isolate the CD-3T vimentin-positive *versus* the CD-5R keratin-positive subpopulations and asked whether one population was selected and/or preferentially influenced by the irradiated microenvironment during growth *in vivo*. We found that both subpopulations were preferentially tumorigenic in irradiated hosts. Thus, differential selection of these particular phenotypes alone does not appear to be responsible for the preferential effect of the irradiated stroma on promoting COMMA-D neoplastic behavior. The two cell populations may, however, interact because tumors arising from each showed distinctly different characteristics from the parent. CD-5R were less tumorigenic and considerably smaller than the parent cell line. In contrast, the CD-3T subpopulations were somewhat more efficient than the parent cell line in establishing tumors, and unlike the parent, were as large in the sham-irradiated host as in the irradiated host.

Our previous studies showed that irradiated mammary gland undergoes rapid remodeling of the microenvironment characterized by changes in ECM and activation of latent TGF-β (25, 36). We tested whether these events were functionally related by treating animals with TGF-β neutralizing antibodies prior to irradiation (37). TGF-β panspecific neutralizing antibody administered shortly before irradiation inhibited collagen III staining in the adipose stroma at 24 h in an antibody dose-dependent manner and blocked collagen III through 7 days after irradiation. Quantitative image analysis demonstrated that exposure to radiation doses of as little as 0.1 Gy elicited a significant increase in TGF-β immunoreactivity, which showed a linear dose response after exposure to 0.1–5 Gy without an apparent threshold of the mammary epithelium. Qualitative evaluation of the reciprocal pattern of latency-associated peptide and collagen III immunoreactivity in the adipose stroma, in contrast, demonstrated a threshold of 0.5 Gy. These data provide functional confirmation of the hypothesis that radiation induces TGF-β activation and implicate TGF-β as a mediator of tissue response to ionizing radiation.

TGF-β orchestrates responses of multiple cell types and has emerged as a key coordinator of tissue response to damage during wound healing, inflammation, and development. Aspects of radiation remodeling parallel those associated with dermal wound healing (46). Ionizing radiation effects on the tissue microenvironment that foster neoplastic behavior may be similar to those observed by other agents that elicit an activated stroma. Experimental animal models have demonstrated that carcinogenesis is enhanced by the activated stroma

induced by wounding (12), overexpression of platelet-derived growth factor (5), or misregulation of stromelysin (41, 47).

Although the particular aspect of the irradiated mammary microenvironment that is stimulating COMMA-D tumorigenic conversion is unknown, the ability of radiation to induce TGF-β activation may indeed play a role (36, 48). We have argued that the role of TGF-β in cancer appears paradoxical in that normal epithelial cells are profoundly sensitive to TGF-β growth inhibition, whereas cancer cells are generally TGF-β resistant (49) because conversion during tumor progression is a critical juncture in establishing malignant behavior of certain epithelia, particularly skin and breast (50). TGF-β has been identified as a cancer-promoting agent in wounds (13, 51, 52), and recent studies have implicated cyclosporin-induced TGF-β in the increased frequency of neoplasms after immunosuppressive therapy (53). TGF-β also mediates phenotypic conversion of epithelial cells to vimentin-positive spindle cells (54). Such a transition is observed in a transgenic keratinocyte model overexpressing TGF-β activity in which the frequency of benign papillomas is suppressed after chemical carcinogenesis, but progression to more malignant spindle cell carcinomas is stimulated (55). In the SCp2 cell line, a subclone of late-passage COMMA-D cells (56), TGF-β treatment stimulates the transition from nontumorigenic keratin-positive cells to tumorigenic, vimentin-positive cells.[6] Studies to determine whether the carcinogenesis-promoting effect of radiation on stroma is attributable to TGF-β activation are under way.

Our hemibody irradiation data support the conclusion that radiation alters the local tissue microenvironment in a way that compromises the restraints imposed by normal stroma on initiated epithelial cells. A role for stroma early in neoplastic progression has also been suggested in hematopoietic malignancies, which have been proposed to result from misregulation of adhesive properties by diseased or genetically aberrant stroma (57). Conversely, the therapeutic benefit of IFN-α in chronic myeloid leukemia has been shown recently to be attributable in part to the reestablishment of cell adhesion signals (15). Greenberger *et al.* (58) proposed a model of indirect γ-irradiation leukemogenesis based on cocultures of heavily irradiated bone marrow stromal cell lines that selectively bound macrophage-colony-stimulating factor receptor-positive unirradiated hematopoietic progenitor cells, resulting in selection of tumorigenic subclones. Additional evidence that radiation effects on stroma alter the behavior of neoplastic cells comes from studies of tumor bed effect, in which stroma that is heavily irradiated prior to tumor transplantation inhibits tumor growth but fosters metastatic behavior (59). Such studies support the conclusion that radiation has global and persistent consequences in terms of stromal function, which in turn can influence the expression of neoplastic potential.

In effect, these experiments demonstrate a bystander-type phenomena *in vivo* in which the products of irradiated cells can significantly alter the phenotype of unirradiated cells. On the basis of these data, we propose that radiation-induced microenvironments are evidence of an additional class of carcinogenic action, distinct from those leading to mutations or proliferation (60). Studies in cell culture indicate that the frequency of morphological transformation can be modulated by restrictive conditions that select for preexisting cell variants (61) and that, conversely, normal cells may actively restrain the expression of the transformed cell phenotype (62). *In vivo* studies by Cha *et al.* (63) show that mammary tumors with *Hras*1 gene mutations from *N*-nitroso-*N*-methylurea-treated rats arose from cells with preexisting *Hras*1 mutations that occurred during early development. Thus, although clearly mutagenic in its own right, *N*-nitroso-*N*-methylurea exposure led to the expansion and neoplastic progression of *Hras1*-mutation containing populations. In our studies, radiation did not directly induce additional mutagenic events because the epithelial cells were unirradiated. We propose that a further action of carcinogens, such as ionizing radiation, is to modify paracrine interactions between the stroma and epithelium in a manner that affects the frequency with which previously initiated cells progress (46). Carcinogen-induced microenvironments are not necessarily mutagenic or mitogenic *per se*. Rather, changes in the microenvironment may promote neoplastic behavior by disrupting normal cell functions that are regulated through cell-cell contact, cell-ECM interactions, and growth factor signaling. Thus, if ionizing radiation induces a microenvironment that modifies restrictive interactions, then it may promote malignant phenotype in manner that is functionally equivalent to the acquisition of additional mutations in the initiated cell. Alternatively, the microenvironment elicited by carcinogen exposure could create novel selective pressures that would affect the features of a developing tumor. Disruption of solid tissue interactions is a heretofore unrecognized activity of radiation as a carcinogen and a novel avenue by which to explore new strategies for intervening in the neoplastic process.

[6] S. Galosy, M. H. Barcellos-Hoff, Z. Werb, and M. J. Bissell, unpublished observations.

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The Influence of the Microenvironment on the Malignant Phenotype

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Abstract

Normal tissue homeostasis is maintained by dynamic interactions between epithelial cells and their microenvironment. As tissues become cancers, there is reciprocal interactions between neoplastic cells, adjacent normal cells such as stroma and endothelium, and their microenvironments. The current dominant paradigm wherein multiple genetic lesions provide both the impetus for, and the Achilles heel of, cancer may be inadequate to understand cancer as a disease process. In the following brief review, we will use selected examples to illustrate the influence of the microenvironment in the evolution of the malignant phenotype. We will also discuss recent studies that suggest novel therapeutic interventions may be derived from focusing on microenvironment and tumor cells interactions.

Introduction

Tissue function is maintained by a dynamic interplay between parenchymal cells and their microenvironment [1]. We define the latter as insoluble extracellular matrix (ECM), stroma consisting of fibroblasts, adipose, vasculature and resident immune cells, and the conventional milieu of cytokines and growth factors (Fig. 1). Epithelial parenchyma are physically separated from stroma by a basement membrane, a highly organized special ECM, whose composition is different from stromal ECM and to which epithelial cells attach. Over the last decade, the cell biology of the complex interactions between epithelial cells and the microenvironment has been firmly established in model systems as critical to maintaining normal, balanced homeostasis [2]. In this review we will discuss evidence that disrupting this balance can induce aberrant cell proliferation, adhesion, function and migration that may promote malignant behavior (reviewed by [3-5]). Furthermore, we will discuss selected studies that illustrate the contribution of the microenvironment to the development of cancer and how manipulating the interaction between cancer cells and the microenvironment may lead to novel therapeutic targets.

Cancer is a multi-step process

The prevailing paradigm for the development of cancer is a multi-step process during which a cell acquires multiple genetic mutations [6-8]. With the advent of molecular genetics, this paradigm has led to a focus on genetic changes in tumors using the tools of modern molecular biology. The central question that has dominated the literature is: how many and what genetic changes are necessary for a cell to become a tumor? With the advent of molecular genetics, this reductionist paradigm has led to many useful discoveries including the critical role of oncogenes and tumor suppressor genes [9]. However, the knowledge gained from cataloging genetic mutations in cancer development is limited. In a step towards functionally categorizing these genetic changes, Kinsler and Vogelstein have classified the genes involved as those that monitor growth ("gatekeepers"),

assisted by genes that indirectly suppress neoplasia by regulating genomic stability ("caretakers"), but have recently also recognized enabling genes, which they refer to as "landscapers", that may affect non-target cells [10, 11]. We prefer, however, to refer to them by the well-accepted term of microenvironmental "effector". Furthermore, tumors are not composed of a single cell type but are composed of multiple cell types. Tumors cannot exist in isolation, and normal cells within the diseased tissue are not idle-bystanders. Rather, normal cells are active participants that shape the frequency and features of tumors. The multi-step genetic modification theory often fails to acknowledge the significance of such forces in tumor development.

With this view in mind, rather than asking how normal cells become cancer cells, one might then ask how are multicellular tissues subverted into tumors? The answer most certainly lies in the cell biology of tissue specificity. The loss of function when tissues are disrupted and cultured as dispersed cells plagued cell culturists for decades [12]. Mammary epithelial cells, hepatocytes, epidermal keratinocytes, Sertoli cells and many other cell types grown on a reconstituted basement membrane form polarized, organized structures characteristic of their organization in vivo [13]. These events are now beginning to be understood in terms of specific proteins affecting cell-cell contacts, cell adhesion and their dynamic interaction with the nucleus via the cytoskeleton [2]. Since cancer has long been postulated to be a problem of development and differentiation (reviewed in [14]), the molecular mechanisms by which cells interact will allow us to cast old problems in new terms.

Selected players in maintaining normal tissue architecture

The basic properties of cellular behavior that define function are growth, morphology, polarity, adhesion, migration, and expression of tissue-specific proteins. These properties constitute the cell phenotype, which is conferred by interaction between its expression of specific genes and its responses to ECM, to neighboring cells and to soluble effectors such as growth factors and

cytokines (Figure 2). Epithelial cells, the major target for adult cancer, exist in contiguous sheets composed of organized, polar cells circumscribed by a basement membrane that separates the epithelium from the stroma. This organization is lost in cancers, along with the integrity of the basement membrane.

There are a vast number of specific molecules originating both locally and distantly that affect cellular phenotype directly and indirectly. Some are mediated via effects on the stroma and others are produced in the stroma. For the purposes of illustrating the role of the microenvironment in neoplasia, we will focus on only two components of this complex network: β1 integrin and transforming growth factor-β1 (TGF-β).

The integrins

The integrins are a large class of transmembrane molecules involved in direct cellular communication with the ECM (reviewed by [15, 16]). The major function of these diverse molecules is to modulate attachment, which is implicated in growth, development, apoptosis, adhesion, migration, and morphology. Integrins form heterodimeric complexes whose cytosolic component directly interact with the cytoskeleton [17]. Recent studies have identified additional functions affecting the action extracellular proteins. An exciting example is the demonstration that αVβ6 integrin binding to latent TGF-β can induce its activation [18]. The β1 integrin subunit, partnered with a variety of α integrins subunits, has been shown to play a critical role in the development of the malignant phenotype [19].

β1 integrins are characteristically expressed on the basolateral surface of epithelial cells in vivo. Cells cultured on conventional tissue culture plastic do not display polarized integrin expression as seen in vivo, but instead, express β1 integrin diffusely over the cell membrane. Non-malignant human mammary epithelial cells cultured inside a reconstituted basement membrane organize into acinar-type structures, and express β1 integrin on the basolateral surface. Tumor cells

either lack β1 or have increased amounts that are not localized correctly [20]. We have used this model (discussed below) to investigate how β1 integrin-ECM interactions act to maintain normal structure, or conversely, how aberrant interactions give rise to a malignant phenotype.

TGF-β

TGF-β is one of a class of myriad soluble polypeptides that coordinate cell function over distances by binding to cell surface receptors. TGF-β is a pleiotropic cytokine with diverse roles in regulating normal tissue growth, development and function. It was initially characterized by its effects on stroma [21], but is also an important mediator of epithelial function and proliferation [22]. Responses to TGF-β include phenotypic changes affecting adhesion, migration, differentiation and cell fate. In general, TGF-β stimulates the production of ECM components while inhibiting degradation, and may also alter integrin expression. It follows that all of these effects in turn can significantly alter cell behavior. TGF-β is abundant in latent forms that circulate or are bound in the ECM. Extracellular modifications activate TGF-β before it can bind to ubiquitous heterodimeric receptors that act through the recently identified SMAD family of signal transduction [23]. Physiologic activity depends on poorly defined extracellular events. Because abundant reservoirs can be rapidly accessed following wounding, ischemia, inflammation and other damage, TGF-β is a prime signal coordinating multicellular tissue processes [24].

Since TGF-β profoundly inhibits epithelial cell proliferation, it is paradoxical that it is also strongly implicated in the development of many cancers of epithelial origin [25]. Cancer cells often have defective TGF-β receptors and signaling pathways yet generate abundant active TGF-β [26]. When tumor cells are no longer sensitive to its growth inhibitory effects, TGF-β provides particular benefit for the tumor by stimulating stromal and angiogenic responses, while inhibiting immune and inflammatory responses.

How does microenvironment affect the development of cancer?

Three stages of tumor development, i.e. initiation, promotion and progression, have been functionally defined in experimental animals. As noted by Pitot, the carcinogenic action of specific agents can be obscured or complicated because endogenous neoplastic events may be influenced by the experimental processes [27]. In recent years, the boundaries between these stages have begun to blur as we begin to recognize the interdependence of genetic change and the microenvironment.

Ionizing radiation

Ionizing radiation is an established carcinogen in many tissues in humans and animals, with a well-defined physical basis for action and well-studied mutagenic capacity. Initiation, the event during which cells acquire neoplastic potential, is presumably a result of genetic mutations in so-called oncogenes or tumor suppressors. The carcinogenic potential of ionizing radiation has long been thought to be based on the initial DNA damage, which when improperly repaired result in mutations, some of which can lead to neoplastic transformation. As a consequence the nucleus has been considered to be the major target of ionizing radiation damage. Nonetheless, over the last decade evidence has accumulated to challenge this dogma and in recent years, has begun to shift the focus away from the mutagenic effects of the initial radiation exposure. The importance of the microenvironment in radiation response is supported by the observation that neighbors of irradiated cells respond with so-called stress proteins as if they were exposed. Little and colleagues have provided a major impetus for change by showing that irradiated cells readily transmit genetic instability by a non-mutational mechanism to their progeny, and, further, that cytoplasmic irradiation can result in mutations (reviewed in [28]). The involvement of cell-cell contact and soluble signals supports the idea that initiation may be a more complex event than previously thought [29]. As a consequence, one may question whether we really understand the nature of the 'initiating' events in carcinogenesis.

In vitro studies

Cell culture studies conducted by Bauer and colleagues underscore the critical importance of environment on the ability of radiation, chemicals and virus to neoplastically transform human and rodent fibroblasts (reviewed in [30]). Bauer demonstrates that increasing the number of cells plated in a classic foci-forming transformation assay results in decreased foci formation, which has also been reported by others. Signals from the non-transformed cells induce the selective ablation of transformed cells via apoptosis, which is mediated in part by the production of TGF-β and the induction of reactive oxygen generation. The remarkable efficiency of this mechanism has been widely obscured by the common practice of using sparse cultures for colony assays. Bauer suggests that normal cells in vivo restrict the ability of initiated cells to express the malignant phenotype by such mechanisms, and that in order to become established tumors, cells must develop resistance to such normal tissue constraints.

In vivo studies

In our own studies we have asked whether the effect of ionizing radiation on the microenvironment promotes the expression of neoplastic potential. We have shown that ionizing radiation produces rapid and persistent remodeling of ECM in mouse mammary stroma (reviewed in [4]). Subsequent studies demonstrated that radiation elicits the activation of TGF-β in situ at remarkably low doses, possibly via the production of reactive oxygen species, which we have shown efficiently converts latent TGF-β to the active complex [24]. To determine whether these events contribute to the known carcinogenic action of radiation, we injected non-tumorigenic mammary cells into the irradiated fat pads of mice. Tumors formed preferentially in fat pads of hosts irradiated up to 14 days prior to transplantation, and only in the exposed half of an animal that was hemi-body irradiated [31]. While a direct functional role for TGF-β has yet to be determined, these data indicate that radiation-induced changes in the stromal microenvironment can contribute to neoplastic

progression *in vivo*. Disruption of tissue interactions is a heretofore unrecognized activity of ionizing radiation as a carcinogen.

Wounding

Expansion of an initiated population, is considered to be the primary action of cancer promoting agents. Experiments in the 1960's by Fisher and colleagues showed that tumors metastasized preferentially to wound sites in parabiotic pairs of animals injected with invasive tumor cells [32]. It has long been recognized that wounding can act as a promoter, apparently by creating a favorable microenvironment for proliferation, a prerequisite for wound repair (reviewed in [33]). Experiments performed with Rous sarcoma virus showed that tumors formed preferentially at sites of injections or at distant wounds. Similarly, several transgenic oncogene models show preferential tumorigenesis at wound sites. TGF-β accumulates at wound sites and when TGF-β injected directly into distal sites in the Rous sarcoma model it was sufficient to promote tumor formation similar to wounding(Figure 3). TGF-β's postulated action is consistent with that of a promoter in that it stimulates mesenchymal proliferation [34].

However, a number of specific agents that have in common the inappropriate activation of TGF-β, including phorbol esters, cyclosporin A (Cyc A) and ionizing radiation, promote the development of epithelial tumors, despite their growth inhibition by TGF-β [35]. Phorbol ester, the well-studied skin promoter, induces expression of TGF-β mRNA while promoting the development of keratinocyte tumors [36]. The paradox of why a growth inhibitor promotes tumorigenesis was addressed using transgenic expression of a constitutively active TGF-β targeted to the epidermis. Ackhurst and Balmain discovered that while TGF-β inhibits the establishment of early benign tumors, it promotes the conversion from benign to malignant carcinomas [37, 38].

Cyc A, an immunosuppressive drug that is used to inhibit organ rejection following transplantation, also influences the establishment of tumors via a TGF-β related mechanism. In

these experiments, lung A549 adenocarcinoma cells exposed to Cyc A in culture develop pseudopodia and membrane ruffling, characteristic of the invasive phenotype, and increase production of TGF-β. Treated cells form tumors more frequently when injected into nude mice. TGF-β neutralizing antibodies abrogated the effect of Cyc A both in cell culture and in vivo (Figure 3).

Future directions: implications for novel therapies

Gene therapy or drugs targeting specific genetic defects, such as p53, are being widely developed as future cancer treatments, with some indications of success. However the notion of targeting an ordered, multi-step sequence of genetic change during progression to malignancy is undermined by pervasive genomic instability in during tumor progression. As a result there are myriad defects not only between individuals and tumor types but also within tumor types and within individual tumors that ultimately limit the scope of cancer genetic-based therapies.

Rather than targeting neoplastic cells, lessons learned from tissue and cancer biology might allow one to design therapies that encourage tissues to reassert their dominance over neoplastic cells. Several novel therapies adopt the strategy of attacking the means by which normal cells are conscripted into the service of tumor formation may provide the means of non-toxic intervention. An excellent example is the strategy to prevent angiogenesis based on the molecular mechanisms by which tumor cells recruit vascular support [39]. Another target is tumor and normal cell induced proteolytic cascades that may generate an 'activated' site similar to wounding that facilitates tumor growth and invasion (reviewed in [40]). The application of protease inhibitors in cancer therapy has reached clinical trials and such mechanisms may underlie dietary components such as soy implicated in prevention. A final example of targeting stromal effectors is the use of retinoic acid in the prevention of lung cancer in smokers. *13-cis* retinoic acid has been shown to decrease the risk of

subsequent tumor formation in individuals at high risk, and was associated with increased expression of a subtype of retinoic acid receptor in the bronchial epithelium [41].

β1 Integrin: possible role in tumorigenic conversion

Progression, during which the malignant phenotype emerges, is usually thought to be irreversible. Evidence to the contrary is found in recent studies of chronic myelogenous leukemia (CML). CML is clinically characterized by unregulated expansion and release of immature cells into the circulation. Interferon-α1 (IFN-α) is used in the treatment of CML, resulting in an initial clinical response in most patients. Normal hematopoietic progenitors become quiescent when cultured in contact with the bone marrow stroma [42]. β1 integrins regulate progenitor-stromal interactions via fibronectin, a primary constituent of the marrow microenvironment which lacks a basement membrane.

Verfaillie and colleagues demonstrated that CML cells are refractory to the normal growth-inhibitory signals present in the normal marrow microenvironment and continue to proliferate. Although CML cells express normal levels of β1 integrin, they do not adhere to bone marrow stroma [43]. However, treatment of a CML cell line, K562, with an antibody that increases the affinity of β1 integrin normalized adhesion to fibronectin in a concentration dependent manner. Increased adhesion significantly inhibited the proliferation of CML cells. Pre-incubation of CML cells with IFN-α increases the ability of cells to adhere to the stroma, and this effect is completely abrogated by the addition of antibodies that block α4 and β1 integrin function.

Furthermore, IFN-α also affects the ability of CML precursors to adhere through indirect effects on BM stroma [44]. Normal stromal feeders pre-incubated with IFNα promote adhesion of CML precursors (Figure 4). Restored adhesion depends on α4β1 and α5β1 integrins, and not directly the result of altered adhesive ligand expression. Thus, IFN-α, which is effective in causing CML remission, induces the restablishment of 'normal' (i.e. non-proliferative) growth regulation.

We have used non-tumorigenic passages of the a human mammary epithelial cell line, referred to as S1 [45], to study the role of microenvironment in the progression of human breast cancer (Figure 5). Although genetically unstable, S1 cells cultured in reconstituted ECM growth arrest and form polarized acinar structures with an organized basement membrane and basolateral distribution of integrins, recapitulating normal in vivo tissue structure. Subsequent tumorigenic passages of these cells (referred to as T4-2) cultured similarly do not growth arrest and form disorganized colonies [46]. T4-2 cells have substantially higher levels of all integrins, especially β1 integrin [20]. Comparative genomic hybridization indicated that both S1 and T4-2 cells harbor an array of genomic rearrangements. Thus it is striking that cultured T4-2 cells can be phenotypically reverted in the presence of β1 integrin blocking antibodies to form organized, polarized acinar structures, even though they remain genetically abnormal. Thus, in this model, the interaction of the cell with its microenvironment via β1 integrin is critical in maintaining a differentiated phenotype, even in the face of genetic aberrations.

Concluding remarks

It is now well-documented that neoplastic cells are influenced by the surrounding microenvironment and vice versa. Such studies support the concept that carcinogenesis is a multicellular process in which the destruction of microenvironment is required for the conversion of a tissue to malignant growth. Molecular analysis of the microenvironment and its deregulation during neoplasia have only now begun in earnest, which should provide insight into the microenvironmental effectors that control cell phenotype. The molecular mechanisms involved in the complex cross-talk between cells and their microenvironment hold great promise as targets for cancer therapy as such research may provide strategies for reverting the malignant phenotype in vivo. Reestablishing interactions between neoplastic cells and the microenvironment should permit

tissue homeostasis to once again dominate cell genotype.

GLOSSARY

Acini: Functional organization of epithelial cells into hollow spheres.

Cyclosporin A: An immunosuppressive drug used to prevent organ rejection following transplantation.

Extracellular matrix: insoluble glycoproteins and glycosaminoglycans to which cells attach via receptors.

Hematopoietic progenitors: The stem cells of the bone marrow that gives rise to the full lineage blood cells.

Integrins: Extracellular matrix receptors that mediate cell adhesion to the Ecm and transduce signals to the cytoplasm and nucleus.

Parabiotic: Animals sharing a vascular system.

Pseudopodia: Extension of the cytoplasm during cell migration, often accompanied by membrane ruffling.

Transformation Assay: In vitro assay of neoplastic potential evidenced by unorganized growth and lack of contact-inhibition.

Transforming growth factor-β: a polypeptide that acts as a signal between cells and tissues.

Outstanding Questions

1. Can changes in the microenvironment precede the progression of neoplastic disease?
2. What features of the microenvironment promote neoplastic disease? Are these tissue specific?
3. Can the microenvironment be targeted therapeutically to prevent cancer?
4. Can manipulating microenvironment reverse cancer?

Figure Legends

Figure 1. Tissue architecture and function is maintained by a dynamic interplay between epithelial cells and their microenvironment. The microenvironment consists of extracellular matrix, fibroblasts, migratory immune cells, and neural elements supported by a vascular network, all within a milieu of cytokines and growth factors. Cells interact with the microenvironment via complex autocrine, juxtacrine and paracrine mechanisms.

Figure 2. Normal tissue architecture and function is maintained by a dynamic interplay between epithelial cells and their microenvironment. The microenvironment plays an important role via multiple cell surface receptors that initiate signals from the microenvironment that affect gene expression in the nucleus.

Figure 3. Interactions with the microenvironment play a critical role in the development of the malignant phenotype as evidenced by the effect of wounding, exposure to ionizing radiation and effects of cyclosporin A. These effects are, in part, mediated by the activation of TGF-b.

Figure 4. Normal proliferation of hematopoietic progenitors is regulated in part by b1 integrin mediated adhesion to the bone marrow microenvironment. CML cells and their progenitors exhibit abnormal adhesion to the bone marrow microenvironment. Treatment with IFN-a results in clinical remission in patients with CML. When CML cells are exposed to IFN-a in vitro, cells will adhere to bone marrow microenvironment with increased affinity, mediated by b1 integrin. Conversely, when bone marrow microenvironment is pre-treated with IFN-a, CML cells will adhere with increased affinity, and this response is abrogated by β1 integrin blocking antibodies.

Figure 5. Pre-malignant (S-1) human mammary epithelial cells cultured in a 3-D matrix will form acini-like structures with an organized basement membrane. When malignant (T4-2) cells, which are derived from S1 cells, are cultured in the presence of b1 integrin blocking antibody, they undergo a phenotypic reversion that resembles the organization and polarity of cultured S-1 cells.

Figure 1

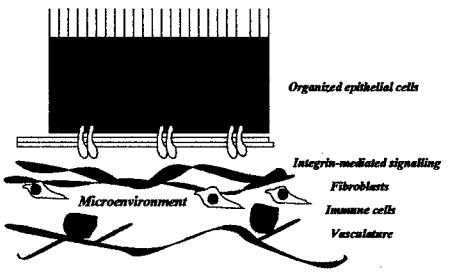


Figure 2

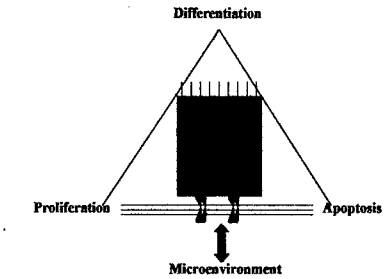


Figure 3

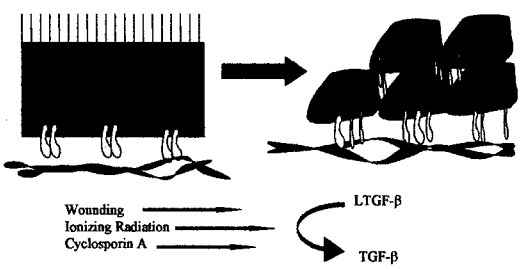


Figure 4

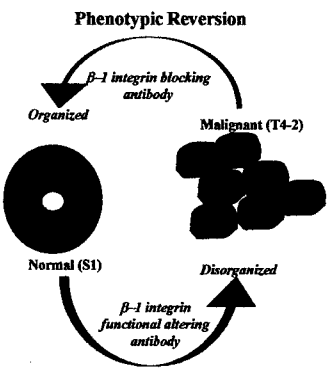
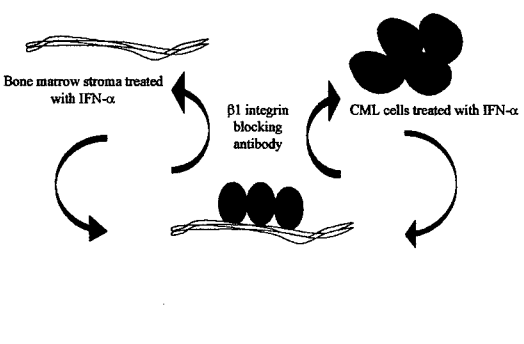


Figure 5



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The Potential Influence of Radiation-Induced Microenvironments in Neoplastic Progression

M. H. Barcellos-Hoff[1,2]

Ionizing radiation is a complete carcinogen, able both to initiate and promote neoplastic progression and is a known carcinogen of human and murine mammary gland. Tissue response to radiation is a composite of genetic damage, cell death and induction of new gene expression patterns. Although DNA damage is believed to initiate carcinogenesis, the contribution of these other aspects of radiation response are beginning to be explored. Our studies demonstrate that radiation elicits rapid and persistent global alterations in the mammary gland microenvironment. We postulate that radiation-induced microenvironments may affect epithelial cells neoplastic transformation by altering their number or susceptibility. Alternatively, radiation induced microenvironments may exert a selective force on initiated cells and/or be conducive to progression. A key impetus for these studies is the possibility that blocking these events could be a strategy to interrupt neoplastic progression.

KEY WORDS: Extracellular matrix; transforming growth factor-β; carcinogenesis; stromal/epithelial interactions; ionizing radiation.

INTRODUCTION

The multistep model of carcinogenesis defines cancer initiation as a genetic change that increases neoplastic potential, and promotion as the series of events leading to proliferation of initiated cells. Promoting agents are thought to increase the probability that a cell will acquire additional mutations necessary for neoplastic progression. Initiated cells have been likened to seeds that require the right soil to flourish. In the breast, the seed arises from the epithelium while the soil is generated by the stroma. Epithelial-stromal interactions, mediated by extracellular matrix and growth factors, generate specialized microenvironments that play a pivotal role in normal tissue development and function (1). It is thought that normal interactions efficiently suppress the expression of the neoplastic phenotype by initiated epithelial cells (2–4). Conversely, abnormal tissue interactions have been postulated to enhance the ability of cells to express the neoplastic phenotype (4–7). It has been postulated that cancer is a physiological response to an abnormal environment (5, 6).

It is well-recognized that tumors may induce changes in the stroma that are conducive to their growth. What if an abnormal stroma were to precede or accompany the early stages of tumorigenesis? In the terminology of carcinogenesis, such a stroma might serve as a promoter in that dysfunction of normal epithelial-stromal interactions could dysregulate growth and increase the probability that a preneoplastic cell progresses to malignancy. On the other hand, in the terminology of development, the microenvironment generated by the abnormal epithelial-stromal interactions may be considered 'permissive' for the growth of certain initiated cells. Both views are based on the tenet that an aberrant stroma may predispose tissue to cancer by increasing the frequency with which an initiated cell proceeds to neoplasia, rather than by increasing the frequency of initiation.

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Ionizing radiation is a complete carcinogen, able both to initiate and promote neoplastic progression; it is a known carcinogen of human and murine mammary glands. Many studies emphasize radiation-induced DNA damage since mutations are believed to initiate carcinogenesis. However the tissue response to radiation is a complex process that is a composite of genetic damage, cell loss and induced gene products. The studies described next demonstrate that radiation elicits rapid and persistent global changes in the mammary gland microenvironment. We postulate that radiation-induced microenvironments can increase the number of transformation susceptible epithelial cells, exert a selective force on initiated cells and/or are conducive to progression (Fig. 1).

The importance of radiation-induced microenvironments is predicated on the central role the microenvironment plays in maintaining cellular function and tissue integrity and conversely, that its disruption leads to the expansion of carcinogen-initiated cells and their progression to neoplasia. Our studies have led us to the hypothesis that *persistent changes in the microenvironment constitute a third class of carcinogenic actions,* distinct from those leading to mutation or proliferative advantage.

MICROENVIRONMENT MEDIATES CARCINOGENESIS

Proximity to a specialized extracellular matrix is a primary mediator of cell phenotype (8). Whereas epithelial cells rest on top of a basement membrane, stromal cells reside below within the interstitial extracellular matrix. It is clear that the microenvironment with which the epithelial cell interacts can dramatically influence its ability to express its differentiated phenotype (Schmeichel *et al.,* (9) in this issue). Several important concepts have been put forward regarding the role of the microenvironment during carcinogenesis:

(1) Normal microenvironments influence the expression and characteristics of neoplastic cells. Transformation in cultured cells has also provided evidence that normal cells influence the expression of the transformed phenotype. Simply altering the culture density profoundly influences the frequency with which transformed cells are morphologically evident: increased density resulted in a decreased number of transformed foci [reviewed in (6)]. At high density normal cells produce growth inhibitors that act in a paracrine and possibly juxtacrine manner to influence

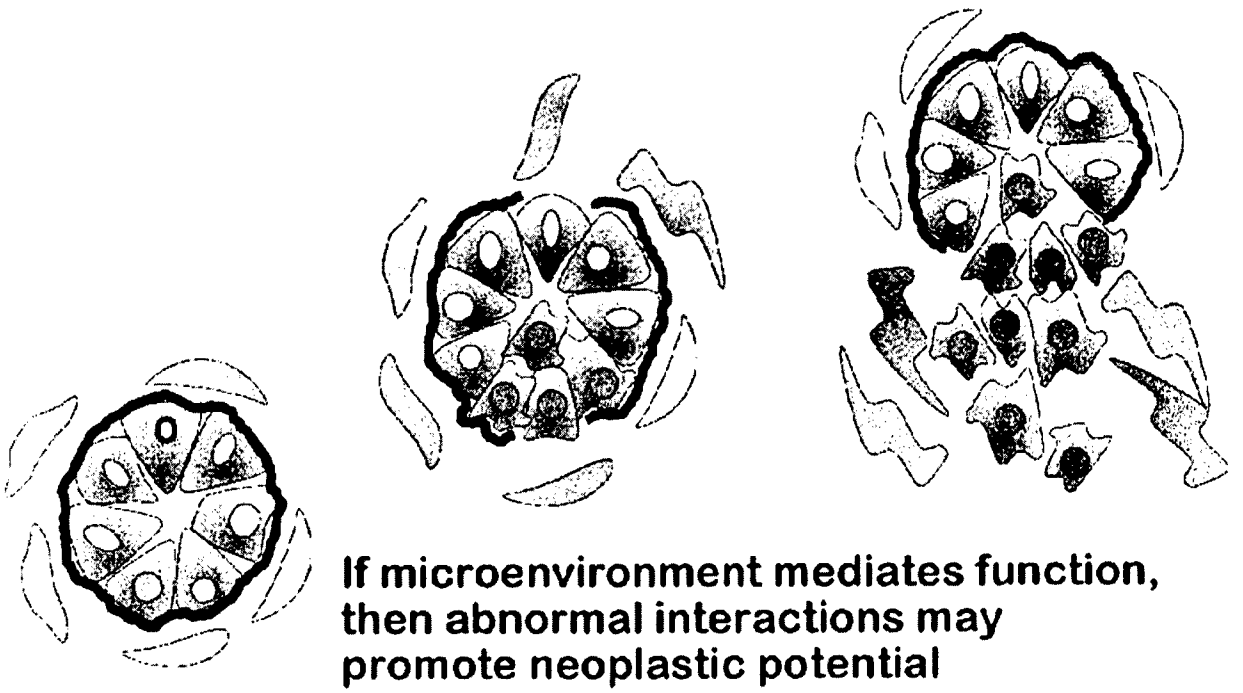

Fig. 1. Alteration of the local epithelial microenvironment contributes to carcinogenesis. We propose that cell containing a oncogenic mutation is constrained by a normal microenvironment, but may proliferate/invade in the presence of an abnormal basement membrane and stroma.

transformed cells. An intriguing and novel concept developed by Bauer states that normal cells eliminate transformed cells in culture via the induction of a short-lived soluble apoptotic signal (10). This mechanism is readily exhibited in cultured cells transformed by chemical, viral, and physical means, is induced by transforming growth factor β, (TGF-β) action on the normal cells and is mediated by production of reactive oxygen species. Bauer postulates that a critical step in the establishment of a tumor is evasion of this regulatory mechanism. Recombination of carcinomas with normal mesenchyme results in varying degrees of differentiation in cancer cells (2, 11). Conversely, Elliot *et al.* (12) demonstrated that markers of malignancy were preferentially expressed when an experimental mammary tumor was grown in the mammary stroma rather than subcutaneously and concluded that the stroma plays a role in modulating the phenotype of malignant cells.

(2) Changes in stromal cell contribution to the microenvironment may be conducive to the expression of preneoplasia by initiated epithelial cells or may promote progression of the preneoplastic cell. This concept is supported by the studies of Sakakura *et al.* (13, 14) in which transplantation of fetal, but not normal adult, fibroblasts into the adult mammary gland induced hyperplastic growth of the normal epithelial elements and rendered the epithelium significantly more sensitive to overt neoplastic transformation by carcinogenic agents. In one example, the introduction of fetal salivary mesenchyme isografted to adult mammary epithelium accelerated the development of mammary cancer (14). Removing and dissociating carcinogen-treated mammary epithelium for subsequent transplantation to mammary fat pad also increased the expression of epithelial dysplasia (15, 16). This result might be interpreted as demonstrating that the disruption of normal stromal/epithelial interactions enhances the expression of preneoplasia.

The unique microenvironment of wounding has also been implicated in the development of certain animal tumors and human cancer [reviewed in (7, 17)]. Interestingly injection of TGF-β can substitute for wounding in Rous sarcoma virus induced tumors (18).

Schor and colleagues proposed that genetic aberrations in fibroblasts may lead to the development of a carcinoma by virtue of abnormal stromal/epithelial interactions on the basis of their demonstration that skin fibroblasts of familial breast cancer patients have abnormal characteristics (19). The phenotype was characterized by migratory patterns similar to fetal cells and was also displayed by fibroblasts from 50% of clinically unaffected first-degree relatives of patients, suggesting that the inherited susceptibility trait affects mesenchymal rather than epithelial phenotype (20). Additional diseases characterized by increased cancer risk have also been correlated with aberrant skin fibroblast phenotypes (21, 22). These observations suggest that the presence of abnormal fibroblasts may precede the emergence of a clinically recognizable malignancy. Such a genetically aberrant stroma is thought to predispose an individual to cancer by increasing the frequency that an initiated cell proceeds to neoplasia, rather than by increasing the frequency of initiation. Alternatively, such alterations in fibroblasts may reflect epithelial cell defects that impact neoplastic potential.

(3) Microenvironment abnormalities may also result from the action of the carcinogen itself. Hodges *et al.* (23) observed that carcinogen-treated stroma recombined with normal bladder epithelium produced neoplastic changes in epithelial morphology. While the effects of chemical carcinogens may be restricted to certain cell types due to the mode of exposure or metabolism, in the case of external radiation, all cells of a tissue are democratically damaged by virtue of energy deposition. Radiation effects on stroma are typically manifested by changes in extracellular matrix composition, as evidenced by fibrosis, which is a well characterized sequella of radiation exposure. Fibroblasts derived from explants of radiation-induced fibrotic skin exhibit persistent phenotypic alterations that do not occur in fibroblasts from normal wound fibrosis (24, 25). These observations suggests that host-independent, inheritable changes occur in stroma as a result of radiation exposure. Since radiation can effectively induce long term changes in tissues, it may also induce particular tumorigenic phenotypes. The production of transin, a protease that degrades basement membranes, by radiation-induced benign skin papillomas correlates with their high rate of conversion to malignancy as compared to chemically induced tumors (26). Since transformed cells both induce basement membrane degradation and are defective in their ability to resynthesize it (27). radiation induced extracellular matrix remodeling may contribute to neoplastic progression by augmenting the destruction of the basement membrane.

RADIATION-INDUCED REMODELING OF THE MAMMARY GLAND

Our studies indicate that tissue responds to ionizing radiation exposure with global remodeling of the

extracellular matrix and induction of potent modulators of epithelial behavior. We observed that a single radiation exposure (whole body, 5Gy γ-radiation) elicited rapid changes in mouse mammary gland extracellular matrix composition and that the changes persisted up to several weeks (28). Although immunolocalization of the basement membrane proteins laminin and collagen IV were unchanged during the week following radiation, marked changes in the periepithelial and adipose stroma were evident. Immunoreactivity of collagen type I in the stromal sheath was reduced by 24 hour and decreased further through day 7. In contrast, collagen III was prominently induced in the adipose stroma, accompanied by increased intensity and abundance of collagen III staining in the periepithelial stroma and septa by day 7. Individually irradiated mammary glands exhibit the same microenvironment changes as those from whole body irradiated animals indicating that these effects are mainly mediated by local factors (E. J. Ehrhart and M. H. Barcellos-Hoff, unpublished). Similar immunolocalization studies in the livers from the same animals indicated that remodeling is a general and rapid consequence of irradiation (M. H. Barcellos-Hoff, unpublished). However, the pattern and composition of the radiation-induced extracellular matrix was tissue specific (e.g., liver showed decreased collagen I and III and increased collagen IV and laminin).

Hyaluronic acid was very rapidly lost from irradiated mammary gland (M. H. Barcellos-Hoff, unpublished data). There was considerable degeneration within one hour of exposure, which became more pronounced with time. Even at 4 weeks post-irradiation there was a striking decrease of detectable hyaluronic acid (Fig. 2). Biochemical analysis of the loss of hyaluronic acid irradiated rat lung over a similar time course and dose (29, 30) are consistent with the rapid and persistent reduction observed in mammary gland. On the other hand, tenascin, which is absent from the ductal tree in adult murine mammary gland (31), was induced in the periepithelial stroma and along the septa (Fig. 3). The tenascin appeared to be deposited in the place of the lost hyaluronic acid in a subepithelial matrix that persisted for at least 7 days.

Although changes in the basement membrane were not observed in γ-irradiated mammary gland (28), we found that laminin localization was affected in mice irradiated with charged particles, e.g., 600 Mev iron, which densely disperses energy as it travels through the tissue, (32). Laminin, a basement membrane glycoprotein, underlines the epithelium when localized with immunostaining. Very shortly after particle irradiation, laminin immunoreactivity showed irregularities, thickening and discontinuities that were evident from 1–9 days. By 14 days these changes had resolved and laminim immunoreactivity appeared normal. The basis for the deterioration of laminin might involve radiation induced proteases. In particular, tissue-type plasminogen activator is induced in a variety of irradiated cells and tissues (33) while plasmin has been implicated in laminin degradation and vice versa (34). Even a transient protease activation by reactive oxygen production (35) could perpetuate extracellular matrix degradation by initiating a protease cascade.

Basement membrane aberrations are characteristic of carcinoma *in situ,* whereas the basement membrane can be disrupted in invasive carcinoma (36). Disruption of basement membrane integrity by either chemical or transgenic means, was shown to promote the expression of mammary tumors (37, 38). The observation that particle irradiation elicits distinct effects on the basement membrane may relate to its enhanced carcinogenic potential, which is an order of magnitude greater than gamma-radiation (39).

A CRITICAL ROLE FOR TGF-β

We have discovered that a key mediator of the rapid response to radiation is TGF-β. The biological activity of TGF-β is constrained by its secretion as a latent complex, consisting of TGF-β non-covalently associated with its processed N-terminal pro-segment, called the latency-associated peptide (LAP). Activation is considered a critical regulatory event for TGF-β function *in vivo* (40, 41). The literature supports a multifaceted and critical role for TGF-β in tissue response to damage via effects on proliferation, programmed cell death (apoptosis), extracellular matrix and growth factor production, chemotaxis and immune response. In many tissue processes TGF-β activation acts as the switch to initiate tissue response to damage. The triple action of TGF-β to inhibit epithelial proliferation, stimulate apoptosis, and mediate extracellular matrix deposition and composition, leads to the conclusion that TGF-β is a key regulator of homeostasis and that its inappropriate activation could impact neoplastic progression.

TGF-β action has been implicated in tissue processes *in vivo* by using neutralizing antibodies or transgenic manipulation leading to overexpression or knockout [reviewed in (42)], but activation has not

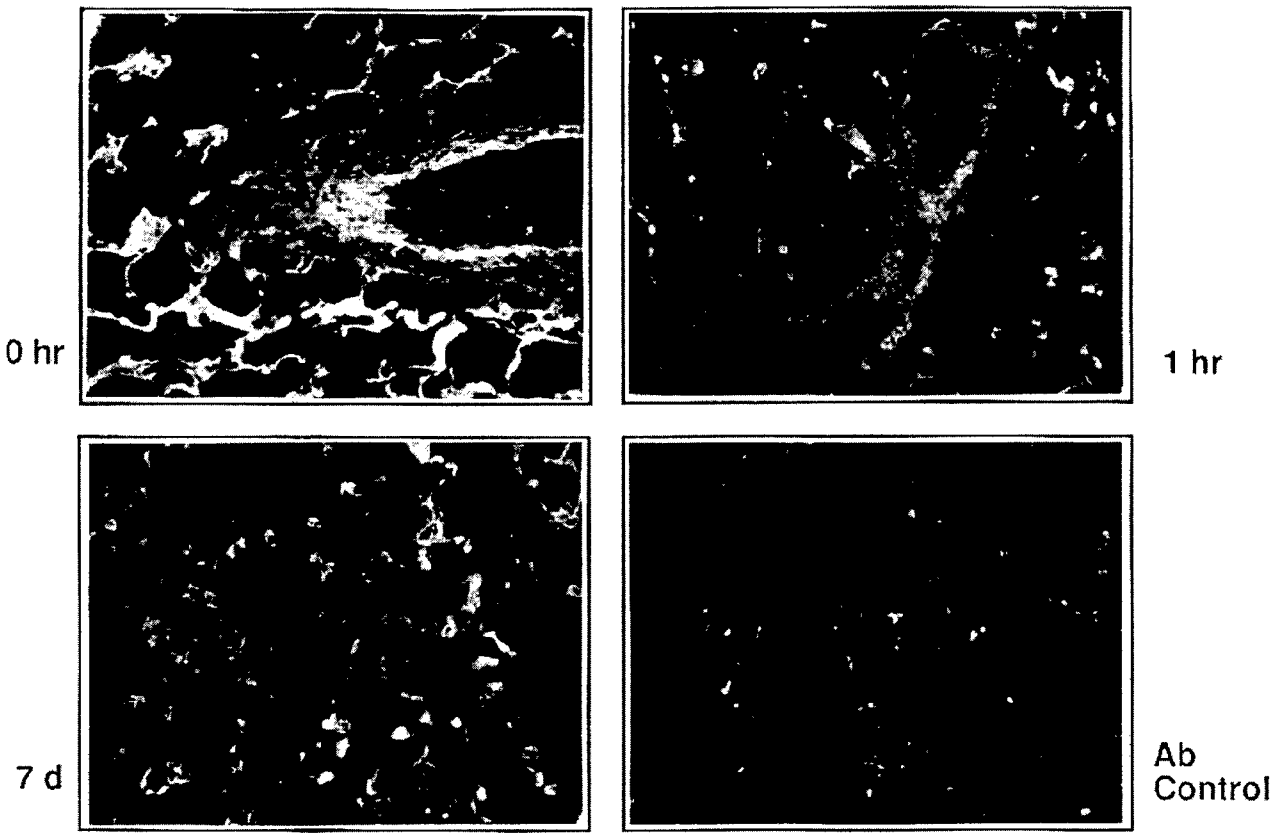

Fig. 2. Hyaluronic acid localization is decreased in irradiated mammary gland. The use of a biotinylated peptide of hyaluronic acid binding protein (kind gift of Dr. Robert Stern) allowed localization of hyaluronic acid in the sham-irradiated (A) mammary gland by fluorescein-labeled strepavidin. Hyaluronic acid detection was decreased as a function of radiation exposure: 1 day (B); 3 day (C), or 7 day (D) after 5 Gy. (40 × original magnification).

been localized specifically or analyzed temporally during carcinogenesis *in vivo*. We developed an immunostaining protocol to selectively detect active TGF-β by immunohistochemistry and to preserve endogenous latent TGF-β (41). We found that latent TGF-β is abundant in normal mammary gland but active TGF-β is restricted to epithelial structures. This pattern of TGF-β immunoreactivity altered significantly one hour after radiation exposure (43). Active TGFβ immunoreactivity was dramatically increased in the epithelium and stroma. This pattern persisted and had not yet returned to the unirradiated levels at seven days after radiation. This suggests that there is a persistent stimulus for TGF-β activation. Activation is detected following exposure to little as 0.1 Gy of γ-radiation (44).

Functional evidence of radiation induced TGF-β activation was obtained by examining collagen III immunoreactivity, a known target of TGF-β (45). Novel collagen III immunoreactivity was evident by 24 hour and colocalized with active TGF-β in the adipose stroma (43). Collagen III mRNA levels were increased and the induction of collagen type III immunoreactivity showed a dose response between 0.5 Gy to 5 Gy, which correlated with a loss of LAP immunostaining (44). Administration of TGF-β neutralizing antibodies shortly before irradiation specifically inhibited the deposition of collagen III in mammary adipose tissue as well as the increase in its mRNA (44). The ramifications of TGF-β activation in radiation carcinogenesis are presently under study; its role in breast cancer is discussed later.

THE TRANSFORMING GROWTH FACTOR-β PARADOX IN BREAST CANCER

The role that TGF-β plays in breast cancer progression remains controversial (46). As recently reviewed by Fynan and Reiss, TGF-β is the most

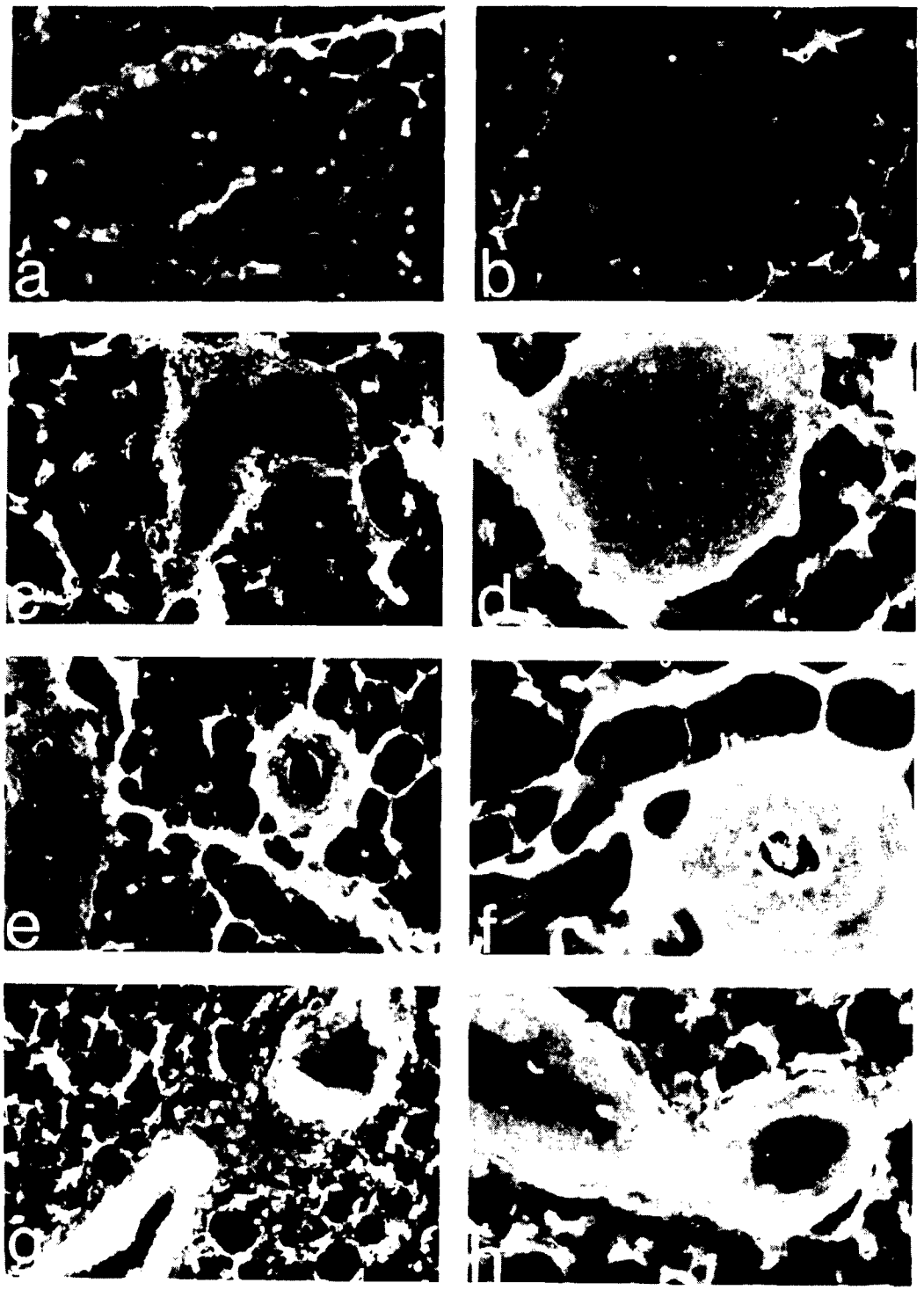

Fig. 3. Tenascin immunoreactivity is induced in irradiated mammary gland. Tenascin was detected using monoclonal antibody 81C6 (the kind gift of Dr. Peter Ekblom) in cryosections from 6-week-old mouse mammary gland prior to (a,b) and 1 day (c,d); 3 day (e,f), or 7 day (g,h) after 5 Gy. Left panel, (20 × (magnification); right panel, (40x original magnification).

potent known inhibitor of cell cycle progression in normal mammary epithelial cells (47). Cell lines derived from nonmetastatic were also growth inhibited in culture. In contrast, virtually all cell lines that give rise to or were derived from invasive tumors *in vivo* are resistant to TGF-β.

If cells are inhibited by TGF-β, then augmenting TGF-β action would be of therapeutic benefit. In support of this contention are numerous observations. Elevated TGF-β mRNA correlates with positive prognosis (48), increased immunoreactivity is elicited by chemotherapy (49), and detectable protein activity correlates with tumor differentiation (50). It has been postulated that TGF-β may be an appropriate target for pharmacological manipulation. Indeed one of the actions of the antiestrogen, tamoxifen, has been postulated to involve the induction of TGF-β activity in the stromal cells (51), as found in human breast cancer patients treated with tamoxifen (49). Supporting the positive correlations in human tissue are studies of cultured cells showing that growth inhibition following exposure to progestin (52), phorbal esters (53), and retinoids occurs via TGF-β. TGF-β neutralization stimulates human breast cancer xenografts (54), while elevated TGF-β immunoreactivity is also observed during treatment-induced regression in animal tumors (55).

However, induction of TGF-β activity may have little negative effect on tumor growth once resistance is acquired. Pretreatment of certain breast cancer cells with TGF-β increases lung metastases (56) and some human breast tumor cell lines, under some conditions, are stimulated by TGF-β (57). Paradoxically, several studies of human disease have found that TGF-β immunoreactivity correlates with disease progression (58), an abnormal stroma (59) and metastasis (60). Increased levels of TGF-β may offer a growth advantage *in vivo* with greater invasive and/or metastatic potential due to suppression of immune surveillance, stimulated angiogenesis, or disruption of normal stromal function [reviewed in (61)].

Since TGF-β resistance appears to be acquired/expressed concomitant with the development of invasive properties and also appears to be associated with increased autocrine production and secretion of TGF-β by breast cancer cells, we proposed a functional link between TGF-β resistance and progression in which the timing of TGF-β activation in carcinogenesis is critical (46). If in the early stages of breast cancer mammary epithelial cells are sensitive to growth inhibition by TGF-β, then TGF-β action is elicited early in carcinogenesis then it is probably a suppressor. Suppression was recently demonstrated by crossing a transgenic mouse that expresses constitutively active TGF-β with mouse with accelerated mammary carcinogenesis due to TGF-α over expression (62). Conversely, over expression of TGF-β dominant/negative type II receptors promote mammary carcinogenesis (63). If, however, TGF-β activity was high at later stages of progression, suppression would be less likely since a heterogeneous population of tumor cells could give rise to TGF-β resistant clones. The differential action of TGF-β during carcinogenesis is best evidenced by recent experiments with transgenic mice overexpressing active TGF-β on a keratinocyte specific promoter (64). In these mice application of the carcinogen, DMBA and a phorbal ester promoter, formation of benign skin tumors was inhibited but progression to invasive spindle cell carcinomas was *enhanced.*

To the best of our knowledge, TGF-β activity is highly restricted in normal tissue in the adult animal. Current models of TGF-β production in cultured cells indicate that activation occurs in a cell-specific, highly regulated and discrete manner (65,66). But if TGF-β is normally latent, how does resistance become a feature of advanced lesions? The observation that radiation, wounding and promoters induce TGF-β activation explains how carcinogen exposure might act as a selective force that can facilitate progression (46). If carcinogen exposure itself induces activation of TGF-β, or induces cells to activate TGF-β, then one would expect selective expansion of cells with mutations that circumvent TGF-β. In this way, carcinogen-modulated nonneoplastic cellular phenotypes contribute to neoplastic progression and to the prevalence of certain phenotypes in the tumor.

THE IMPACT OF MICROENVIRONMENT ON THE MULTISTEP CARCINOGENESIS MODEL

Quantitative studies in mouse and rat mammary gland and rat trachea have demonstrated that the number of cells initiated by either physical or chemical carcinogen exposure far exceeds the number of tumors that develop (4, 67–70). Further, not all mutagens are carcinogens and even a high frequency of mutation is insufficient for efficient tumor formation. For example, Vogelstein and colleagues identified a subset of hereditary nonpolyposis colorectal cancer patients who carry a germline mutation resulting in a mismatch DNA

repair defect that is the basis for hypermutability (71). Even though nontumor cells carry high levels of mutations, individuals develop normally, suggesting that mutations *per se* are insufficient for tumorigenesis and that some other stimulus is necessary for cancer development (72).

On the other hand, disruption of stromal/epithelial interactions may provide the stimulus for cells to move partially into the neoplastic pathway even without an apparent mutagenic event (38). As such, the effects of carcinogens on gene expression by nonneoplastic cells that lead to an altered microenvironment may be considered as a third class of carcinogenic action distinct from mutation or mitotic stimuli. Carcinogen-induced changes in the microenvironment may either be conducive to progression or exert a selective force or both, but are not mutagenic or mitogenic *per se*. Rather, conducive changes in the microenvironment are effective because they disrupt inhibitions that normal cells effect through cell-cell contact, cell-matrix interactions and growth factor production. In essence, the carcinogen-induced microenvironments remove obstacles that the initiated cell would require additional mutations for it to circumvent. Thus phenotypic changes induced by noninitiated cells following carcinogen exposure could permit initiated cells to elude a normal physiological barrier to expansion.

CONCLUSIONS

We have reviewed the character of the irradiated mammary gland microenvironment and proposed that carcinogen exposure of nonneoplastic cells can impact the progression of initiated cells in novel ways. The following predictions are the result of these studies: *If carcinogen exposure induces a microenvironment is restrictive, then it should affect the features of the resulting tumors.* Selection is readily demonstrated in cell culture. When cells are placed under restrictive conditions, by growing in the presence of a soluble factor or a physical barrier in invasion assays, some cells escape that particular restriction. When the selective pressure in nonmutagenic, it is presumed that heterogeneity within the population is necessary. Carcinogen-induced TGF-β activation may impose a restrictive condition. If so, TGF-β resistant cells should predominate in the resulting tumors. An example might be the mutations in the type II TGF-β receptor recently reported to be a frequent occurrence in colon cancer cells with the hypermutability phenotype [72]. It is not known why selection for escape from TGF-β inhibition occurs but one source of selective pressure could be the tissue microenvironment if the promotion process in human colon elicits TGF-β activation, as would occur with wounding or infection. Our current experiments are testing whether radiation-induced activation of TGF-β produces such a restrictive microenvironment in the breast.

If a carcinogen-induced microenvironment is conducive, the frequency of neoplastic progression should be affected. There are a number of types of conducive environments. Carcinogen-induced activation of proteases that degrade the basement membrane might allow initiated cells to escape its restraint without the need to gain further mutations. Such a mechanism might be responsible for the tumors that arise in the transgenic mouse model that over expresses an activated form of the extracellular matrix protease, stromelysin (38). The 92-kDa gelatinase/collagenase, which has been linked to the metastatic phenotype in transformed cells (74), is transiently induced in irradiated mammary gland (L. Lund and M. H. Barcellos-Hoff, unpublished), as well as other tissues (33). Densely ionizing radiation causes discontinuities in mammary gland basement membranes, which could facilitate invasion or promote growth (32). The loss of hyaluronic acid and gain of tenascin in irradiated tissue may be conducive to mammary dysplasia, as demonstrated by Silberstein and Daniel using implants containing hyaluronidase (75). Thus radiation could produce a conducive microenvironment by altering the expression of proteases, disrupting the basement membrane or altering the expression of extracellular matrix proteins. More investigation is needed to determine the extent to which these effects contribute to the known carcinogenic action of ionizing radiation.

If the microenvironment induced by carcinogens can shape the features (selection) and frequency (conducive) of neoplastic phenotypes, then the carcinogen 'fingerprint' may be envisioned as being built by first laying a foundation of genotypic alterations that expand in the context of a microenvironment that is the result of carcinogen-induced phenotypic change. Understanding this aspect of carcinogenesis is important since certain microenvironment alterations might be amenable to modulation. Such manipulation in turn provides the means to modify cancer progression.

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How Do Tissues Respond to Damage at the Cellular Level? The Role of Cytokines in Irradiated Tissues

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The capacity of ionizing radiation to affect tissue function, control tumor growth and elicit pathological sequelae has been attributed in great part to its effects on cellular DNA, which, as the transmitter of genetic information, can both register damage and perpetuate it. Nonetheless, multicellular organisms function as the result of the cooperation of many cell types. What then occurs when individual cells are damaged by ionizing radiation? Is tissue response a sum of cellular effects such as cell death and DNA damage? Or does the tissue respond as a coherent unit to the damage of its parts? In this paper, data in support of the latter model that indicate a role for cytokines, in particular transforming growth factor β1, as critical components of extracellular signaling pathways that mediate tissue response to radiation will be reviewed. The key to manipulating the consequences of radiation exposure lies in understanding the complex interplay of events initiated at the cellular level, but acting on the tissue.

INTRODUCTION

The organization of multicellular organisms into purpose-specific tissue is obtained through differential expression of the genome. The underlying diversity of multicellular tissue organization is seen in the heterogeneous temporal and spatial patterns of gene and protein expression that are revealed at the level of the cell by *in situ* hybridization and immunohistochemistry techniques. Understanding how a cell differentiates itself from its neighbors, yet is able to perform as part of a unit, is fundamental to understanding how tissues carry out complex organ functions. A cell receives information about what it is and how it should behave from its microenvironment, which consists of other cells, insoluble extracellular matrix (ECM)[1] proteins, soluble hormones and cytokines (*1*, *2*). Cells are linked to their microenvironment via membrane receptors that initiate signaling cascades, which in turn have an impact on cell behavior and gene expression (*1*, *2*).

In response to damage, the flow of information both locally between cells and tissues and distantly between organs is mediated in large part by cytokines (*3*). Tissue pathology and organ failure can arise from the lack of orchestrated communication between cells and among different cell types. When wounding disrupts tissue integrity, a well-defined and coordinated series of cell behaviors aimed toward re-establishment of homeostasis is initiated. Tissue trauma releases blood, which induces clotting and the concomitant release of cytokines that elicit sequential inflammation, formation of granulation tissue and healing. In contrast, exposure to ionizing radiation at environmental, diagnostic or therapeutic doses has little in common with this type of wounding since tissue integrity is not rapidly compromised. Radiation damages individual cells; thus one can argue that the response to radiation is the sum of the responses of individual cells, such as cell death. However, emerging data indicate that tissues respond to radiation in a coordinated and multifactorial fashion and that radiation exposure ultimately compromises tissue integrity by altering the flow of information among cells. Understanding this response is the key to prevention of adverse sequelae.

Evidence for a radiation-induced program of gene expression directed from irradiated cells to their microenvironment begins with studies of gene expression in cultured cells. Intracellular signal transduction pathways that mediate cellular responses to ionizing radiation are being defined in great detail (*4*). Certain molecular elements are common to many stress responses, in particular oxidative stress, and include the induction of early response genes (*5*, *6*) and up-regulation of DNA repair systems (*7*). However, a number of genes that contribute neither to the regulation of the genome nor to DNA repair are induced in a variety of cell types. Tissue-type plasminogen activator (tPA) is induced in fibroblasts of human (*8*) and murine origin, normal or transformed (*9*), glial cells (*10*), endothelial cells (*11*), brain stem (*12*) and lung (*13*). The generality of tPA gene activation points to a conserved response whose purpose in single cells has remained obscure. Induction of collagenase has also been observed in a variety of cell culture models

[1]Abbreviations used: ECM, extracellular matrix; LAP, latency-associated peptide; PAI-1, plasminogen activator inhibitor 1; TGF-β, transforming growth factor β; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator.

(*10, 14, 15*). Cytokines such as tumor necrosis factor α (*16*), α-interferon (*17*), transforming growth factor β (TGF-β) (*18*) and fibroblast growth factor 2 (*19*) are transcriptionally activated in irradiated cultured cells. The patterns of gene induction in tissues (*20, 21*) are further complicated by the contribution of multiple cell types, inflammatory cells and secondary effects. The benefit or contribution of the products of such genes to the overall response to radiation is as yet unknown.

If the unit of function is taken into account, i.e. tissue, it becomes self-evident that many of these events are likely to be directed to the good of the whole, rather than the part, i.e. the cell. Thus the autocrine role of these induced proteins may be outweighed by their juxtacrine, paracrine or endocrine functions. The cytokine cascade described in the lung in which radiation-induced cytokines recruit inflammatory cells that then influence other cells to contribute further to the production of cytokines (*22*) underscores the complexity of the biological response networks. It is reasonable to expect that predicting the effect of these gene products depends not only on the recipient cell type, but also on the context in which these extracellular signals are conveyed.

COMPOSITION OF THE MICROENVIRONMENT

The microenvironment encompasses soluble intercellular signaling molecules, along with the acellular ECM and cell–cell interactions, both within and between tissue compartments such as the stroma and epithelium (Fig. 1). Cytokines are soluble polypeptides released by cells to act nonenzymatically at femtomolar to nanomolar concentrations on themselves (autocrine), adjacent cells (juxtacrine), neighboring tissues (paracrine) or distant tissues (endocrine) (*3*). Cytokines often attach to the ECM in which cells are embedded. Information conveyed by interactions with the microenvironment is assimilated and integrated by cells in a manner that is currently poorly understood to produce selective gene expression (*23*). Cellular phenotypes result from the selective expression of the genome and in turn modify the microenvironment through differential production of growth factors, ECM and other secreted products. The challenge is to determine how all this information is integrated by cells so that the programs of development and differentiation and responses to injury can be implemented in a coordinated fashion by individual cells within multicellular tissues.

Cytokines are predominantly produced locally and alter cell function by binding to specific cell-surface receptors, which use phosphorylation and other mechanisms intracellularly to initiate signals that change gene expression. In addition to synthesis and receptor binding, the efficacy of many growth factors is controlled by post-translational modifications, defined protein interactions and extracellular proteolysis (*24*). The activity of any single cytokine also depends on the context in which it is received. Cellular response is influenced by differentiation status, the array of cell-surface receptors, the types and mechanisms of attachment to the ECM, and the current signal input. As a result, the specific bioactivities in cell culture that are often used to classify cytokines may be misleading and the effects of cytokines *in vivo* may appear diverse, unpredictable or paradoxical.

Proximity to a specialized ECM is a primary mediator of cell phenotype (Table I). Whereas epithelial cells are in contact with a basement membrane, stromal cells reside below, within the interstitial ECM. Both stromal and epithelial cells contribute to the composition of the basement membrane. Fibroblasts can induce the assembly of basement membrane complexes in culture, even in the absence of their epithelial counterparts (*30*). Sulfated glycosaminoglycans, produced by the epithelium, and tenascin, produced by the stroma, are basement membrane ECM proteins that affect proliferation

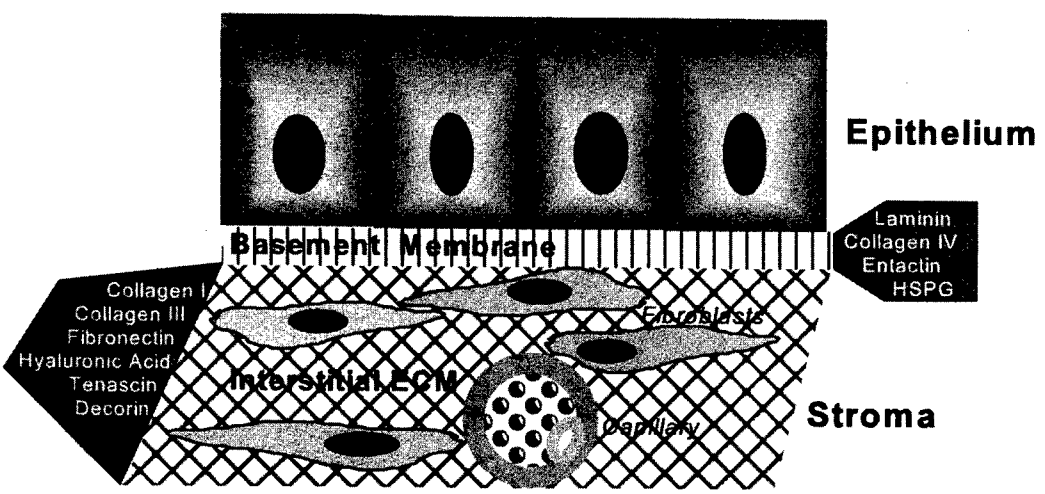

FIG. 1. Schematic of the components of epithelial tissues. Extracellular matrix of epithelium is the basement membrane which is composed of specific proteins. The basement membrane separates the epithelium from the stroma. Stromal cells such as fibroblasts produce and reside in a specialized interstitial matrix. Roles for the various components of ECM are summarized in Table I.

TABLE I
Major ECM Molecules

Molecule	ECM type	Function	Reference
Collagen I	Interstitial	Major structural protein of the body, major product of fibroblasts; increased in fibrosis	*25*
Collagen III	Interstitial	Contributes to ECM integrity and flexibility; increased in fibrosis	*25*
Collagen IV	Basement membrane	Major structural protein of the basement membrane	*25*
Laminin	Basement membrane	Major functional protein of the basement membrane; supports growth and differentiation	*26*
Hyaluronic acid	Interstitial	Major glycosamionoglycan, found at stromal/parenchymal interface, facilitates migration; growth factor reservoir	*27*
Fibronectin	Interstitial	Abundant glycoprotein; important mediator of cell function	*28*
Tenascin	Interstitial/basement membrane	Glycoprotein; developmentally regulated; mediates cell migration and proliferation; increased in tumors	*29*

of epithelial cells (*31*, *32*). The interstitial ECM, composed of collagen types I and III and fibronectin, is synthesized by fibroblasts as a collagenous sheath separating epithelia from other tissue compartments. Studies of cells in culture suggest that one role of collagen substrata is to stabilize the basement membrane produced by epithelial cells (*33*, *34*). It may serve a similar role *in vivo* since inhibition of collagen deposition *in vivo* causes the developing secretory mammary gland to involute (*35*).

The ability of the ECM to bind growth factors and serve as a reservoir is a major facet of its role in differentiation and development. Growth factors are deposited in highly stable forms along with the ECM by cells in culture (*36*). A physiological role for ECM may be to sequester and concentrate growth factors in proximity to cell membranes since basal surfaces of epithelium express receptors for growth factors. Growth factors also stimulate the production of ECM. Epidermal growth factor appears to enhance the accumulation of ECM that serves to stimulate epithelial cell growth (*37*). Silberstein and Daniel (*38*) have suggested that TGF-β contributes to tissue morphogenesis through its effects on deposition of stromal ECM. ECM may in turn modulate the production of growth factors (*39*, *40*). Conversely, studies in cultured cells indicate that the association of cells with an ECM alters their response to growth factors (*37*, *41*, *42*) and hormones (*43*). Thus perturbations in the interaction between the ECM and epithelium could also alter the production of, concentration of and/or response to growth factors.

This brief synopsis hardly does justice to the breadth of research supporting the view that components of the microenvironment are critical conduits of information necessary for function and homeostasis (for review see ref. *2*). The differentiated state of epithelial cells is poorly maintained in culture without an intact basement membrane (*44*), and *in vivo* development is disrupted when ECM deposition is inhibited (*31*, *35*). Thus disruption of this critical interaction by a variety of means negatively affects cell phenotype and promotes aberrant behavior, particularly neoplasia (*45*). In this context, experimental studies that have identified rapid alterations in the composition of the basal lamina (*46*, *47*) and changes in the quantity and composition of the interstitial ECM (*48*, *49*) gain new relevance, although the effects of such changes on tissue function have not yet been appraised.

RADIATION-INDUCED MICROENVIRONMENTS

Recent studies in this laboratory indicate that exposure to ionizing radiation rapidly induces global remodeling of the ECM and generates cytokines that are potent modulators of cell behavior. We observed that a single radiation exposure (0.1–5 Gy whole-body γ radiation) elicits rapid changes in the ECM of the mouse mammary gland that persist for several weeks (*49*). Even though the events described below occur very soon after irradiation, and thus may be adventitious rather than instrumental in the outcome of radiation exposure, they represent a fundamental process initiated by radiation exposure that broadens our understanding of tissue and radiation biology. It is unclear from the nature of our current studies which of these events promote tissue dysfunction or alternatively may be necessary for tissue recovery in an as yet unappreciated way. However, a variety of experimental techniques ranging from neutralizing antibodies to transgenic mice to recombinant proteins are available to test the impact of specific events in radiation responses.

We have identified a remarkably dynamic process of remodeling of the ECM using protein immunolocalization and fluorescence detection to evaluate tissue compartments individually and to map changes in the character and composition of the irradiated microenvironment.

Marked deviations from sham-irradiated controls are rapidly evident in the periepithelial and adipose stroma after a single whole-body exposure to 5 Gy of γ radiation. Immunoreactive collagen type I in the fibrous periepithelial stroma was reduced by 24 h and decreased further through day 7 (*49*). In contrast, collagen III in the periepithelial stroma and tissue septa increased by day 7. Interestingly, collagen III was prominently induced in the adipose stroma from 1 to 3 days postirradiation but had resolved by 7 days, indicating that irradiation elicits cell type-specific responses in related tissue compartments, in this case the adipose stroma compared to the fibrous stroma. Other components of the ECM, such as the basement membrane proteins laminin and collagen IV, were unchanged during the week after irradiation. Individually irradiated mammary glands exhibit the same changes in the microenvironment as those from whole-body-irradiated animals, indicating that these effects are mediated mainly by local factors (Ehrhart and Barcellos-Hoff, unpublished results). In addition, total-body doses of 5 Gy and less do not alter vascular integrity or elicit a notable inflammatory response.

Examination of the ECM of livers from the same animals indicates that remodeling is a general consequence of irradiation, but that the pattern and composition of the radiation-induced remodeling of ECM are tissue-specific (Fig. 2). The liver is 90% hepatocytes that reside proximal to ECM-producing lipocytes, which in turn are adjacent to sinusoidal endothelial cells. After exposure to ionizing radiation (5 Gy whole-body γ rays), remodeling of the microenvironment is rapid and global, as it is in the mammary gland, but the character and composition are distinct. While in the mammary gland the decrease in collagen type I and III in the periepithelial stroma is accompanied by induction of collagen III in the adipose stroma (*49*), in irradiated liver, collagen type I and III are lost rapidly (>1 day), and this persists for more than 7 days (Fig. 2A–C). On the other hand, collagen type IV, which is unaffected in irradiated mammary gland (*49*), is strongly induced in the perisinusoidal space in irradiated liver (Fig. 2D–F). These data suggest that tissue type plays a prominent role in determining the character and composition of radiation-induced remodeling.

A particularly interesting change is found at the interface of the stroma and epithelium of the mammary gland. Hyaluronic acid was rapidly lost from irradiated mammary gland (*50*). Considerable loss was evident within 1 h of exposure and became more pronounced with time. Furthermore, at 4 weeks postirradiation there was still a striking reduction of detectable hyaluronic acid. Biochemical analysis of hyaluronic acid in irradiated rat lung at the same dose and over a similar time course shows a similar rapid and persistent reduction (*46*, *47*) as that observed histochemically in the mammary gland. On the other hand, tenascin, which is absent from the ductal tree in the adult murine mammary gland (*51*), was induced in the periepithelial stroma and along the septa (*50*). Tenascin was induced at the same interface between epithelium and stroma in which hyaluronic acid was lost. Tenascin organized into a subepithelial matrix that persisted for at least 7 days. Both tenascin and hyaluronic acid are considered important for morphogenesis by facilitating migration of cells and are developmentally regulated. Interestingly, the loss of hyaluronic acid and induction of tenascin are individually associated with neoplastic cell behavior and tumor growth (*52*).

Radiation quality, specifically iron-particle irradiation, also affects the composition and character of remodeling of the microenvironment. Immunoreactivity of laminin was specifically affected in mice irradiated with 600 MeV/nucleon iron particles (*53*). Laminin is a basement membrane glycoprotein to which epithelial cells adhere via integrins (*54*, *55*). Very shortly after exposure to particle radiation, immunoreactivity of laminin showed an irregular appearance (i.e. smudged, thickened and discontinuous) that was evident from days 1 through 9, but had resolved by 14 days. The alteration of immunoreactivity of laminin may involve radiation-induced proteases since laminin can be degraded by tPA (*56*), which is

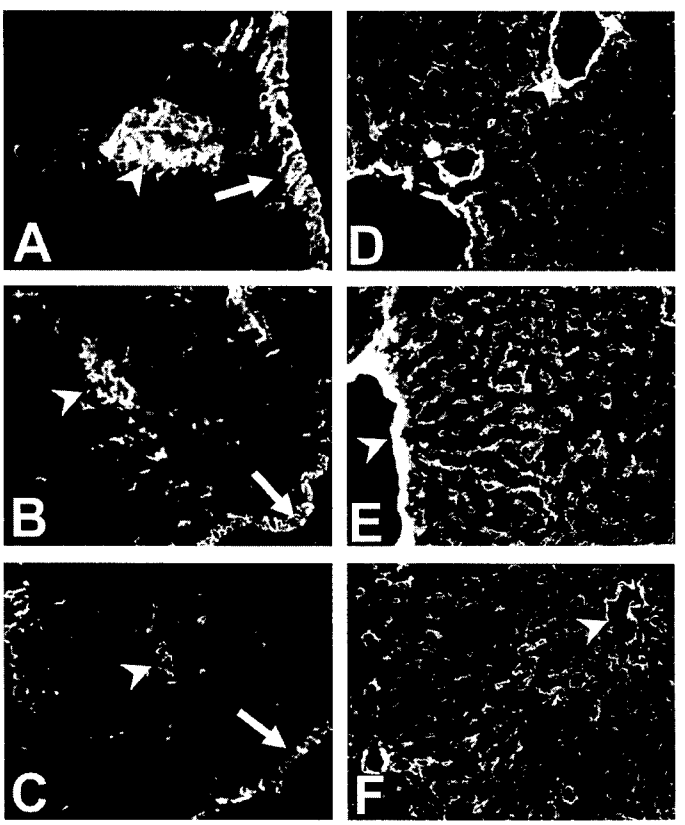

FIG. 2. Immunostaining of collagen type III (panels A–C) and collagen type IV (panels D–F) in liver from unirradiated animals (panels A, D) or 3 (panels B, E) and 7 days (panels C, F) after animals received 5 Gy of whole-body γ radiation. Collagen type III immunostaining decreased progressively at the capsule (arrows) and periportal triad (arrowheads) as a function of time postirradiation. Collagen type IV was increased at 3 days postirradiation in the space adjacent to sinusoids and around periportal tracts (arrowheads) but had returned to normal levels by 7 days. Staining was conducted as detailed in ref. (*44*). Original magnification was 200×.

rapidly induced in a variety of irradiated cells and tissues (*9, 10, 57*). Degradation of the ECM in itself is also a stimulus for release of plasminogen activators (*58*). Induction of a protease activity by radiation could thus be perpetuated by a self-amplifying cascade in tissues. Other events such as induction of collagen III and tenascin were evident but delayed compared to responses to γ radiation (*53*). The influence of radiation quality on alterations in the microenvironment may provide some insight into the mechanisms that mediate remodeling of the ECM.

Mechanisms

TGF-β1 is the founding member of a large family of polypeptide growth factors that are abundant extracellularly in a latent form. TGF-β is an important regulator of the stability and composition of ECM (*59–61*). Exogenous TGF-β stimulates expression of collagen I and glycosaminoglycan in the developing mouse mammary gland (*61*). It has been suggested that production of ECM might act indirectly to reinforce growth inhibition (*62*) and that TGF-β promotes this effect (*38*). In addition, TGF-β is expressed during tissue remodeling involving apoptosis (*63*), and exogenous TGF-β induces cell death both in cultured cells (*64*) and in tissues (*65*). The triple action of TGF-β to inhibit epithelial cell proliferation, stimulate apoptosis and mediate deposition and composition of ECM suggests that TGF-β is a key regulator of homeostasis and that its inappropriate activation would have an impact on a variety of tissue processes.

We discovered that TGF-β is a key mediator of the rapid remodeling of ECM after irradiation. TGF-β has a multifaceted role in the response of tissue to damage through its effects on proliferation, programmed cell death (apoptosis), production of ECM and growth factors, chemotaxis and immune response (*66*). The biological activity of TGF-β is constrained by its secretion as a latent complex, consisting of TGF-β noncovalently associated with its processed N-terminal pro-segment, called the latency-associated peptide (LAP). Release from LAP is a prerequisite for TGF-β to bind to its cell surface receptors. Events associated with release are called activation, which is considered to be a critical regulatory event for the function of TGF-β *in vivo* (*67, 68*). Activation releases TGF-β, which acts as the switch to initiate the response of tissue to damage in several physiological processes, in particular inflammation, wounding and healing (*69, 70*).

Activation has not been localized specifically or analyzed temporally *in vivo*. TGF-β activity has been implicated in certain events *in vivo* by using neutralizing antibodies, transgenic manipulation, leading to overexpression, and loss in "knockout" mice (reviewed in *71*). We developed an immunostaining protocol to preserve endogenous latent TGF-β and to discriminate between latent and active TGF-β by immunohistochemistry (*68*). We found that latent TGF-β is abundant in the normal mammary gland but that localization of active TGF-β is restricted to the epithelium. This pattern of TGF-β immunoreactivity is altered significantly after irradiation (*72*). Soon after irradiation (1 h), TGF-β immunoreactivity was increased dramatically in the epithelium and stroma concomitant with decreased LAP immunoreactivity. This reciprocal shift in immunoreactivity is consistent with a process in which LAP is degraded after release of TGF-β, which in turn reveals previously masked regions of TGF-β, as would occur with activation. This pattern persists for more than 7 days after irradiation, suggesting that there is a chronic stimulus for activation of TGF-β.

Functional evidence of radiation-induced activation of TGF-β was obtained by administration of TGF-β-neutralizing antibody to animals prior to irradiation. Collagen III is a known target of TGF-β (*60*). Collagen III mRNA in total mammary gland RNA was increased by 16 h postirradiation. Novel collagen III immunoreactivity was evident by 24 h postirradiation, co-localized with active TGF-β in the adipose stroma (*72*), and showed a dose response between 0.5 and 5 Gy that correlated with loss of LAP immunostaining in the adipose stroma (*73*). Administration of TGF-β-neutralizing antibodies shortly before irradiation specifically inhibited the deposition of collagen III in mammary adipose tissue as well as the increase in its mRNA (*73*). Radiation is the first exogenous stimulus known to cause activation of latent TGF-β *in situ* (*72*).

Activation of latent TGF-β showed a linear dose response to γ-radiation exposures of 0.1 to 0.5 Gy in the mammary epithelium, while there was a threshold of 0.5 Gy in the adipose stroma (*73*). The dose threshold identified at 0.5 Gy could be a true threshold or could be due to the patchy, open nature of the adipose stroma and an inability to observe subtle changes in the immunoreactivity pattern. However, we detected loss of LAP in the adipose stroma 1 h after exposure to 0.1 Gy (Barcellos-Hoff, unpublished observation), suggesting that there is a difference in susceptibility to activation at 3 days postirradiation. This threshold suggests that mechanisms leading to persistent activation may be tissue- or cell type-specific. The sensitivity of activation of TGF-β to exposure to low-dose radiation and the rapidity with which activation is detected (*72*) suggest that the molecular mechanisms by which ionizing radiation activates latent TGF-β *in situ* may lead to further insight into its biology. Since remodeling of collagen type III showed a distinct character and chronicity in the periepithelial stroma compared to the adipose stroma, different cell types may exhibit specific responses to TGF-β (*49*). The lack of a demonstrable qualitative or quantitative threshold in mammary epithelium suggests that activation of latent TGF-β is one of the most sensitive indices of tissue response to ionizing radiation.

Proteases are thought to be the most common mechanism of activation of TGF-β *in vivo*. Endothelial cells and smooth muscle cells act cooperatively via generation of plasmin to activate latent TGF-β (*74, 75*). Activation of latent TGF-β is mediated by tPA, urokinase-type plasminogen activator (uPA) and plasminogen, and is regulated by the feedback of TGF-β-induced plasminogen activator

inhibitor 1 (PAI-1) (*74*). tPA mRNA and protein are induced by radiation exposure in a variety of cell types (*9*, *76*), and elevation of plasminogen activator activity has been linked to radiogenic fibrosis in rat lung (*13*). Preliminary data from RNase protection assays indicate that tPA and PAI-1 are induced severalfold in irradiated mammary gland (Barcellos-Hoff and Carroll, unpublished results). Dual immunolocalization of tPA and PAI-1 in the irradiated mammary gland suggests that mammary epithelial cells express much more tPA than surrounding stromal cells, while PAI-1 is preferentially induced in the irradiated adipose stroma (Fig. 3). The net effect could contribute to differential dose sensitivity of latent activation of TGF-β in each tissue compartment if plasmin is the mechanism of persistent activation.

Consequences

TGF-β regulates production and degradation of ECM and is a potent modulator of angiogenesis, immune responses and inflammation (*66*). The myriad effects of TGF-β during tissue response to damage suggest that TGF-β acts as an extracellular linchpin, restrained as a latent complex but poised to execute multiple functions when events lead to activation. Several studies have now demonstrated increased expression of TGF-β in irradiated tissue. TGF-β immunoreactivity is elevated in irradiated human and animal tissues that develop fibrosis, which prompted the hypothesis that TGF-β mediates late tissue reaction (*77–80*). A major effect of TGF-β is to modulate the quantity and composition of the ECM by increasing gene expression in the ECM and inhibiting proteases involved in the degradation of ECM. The role of TGF-β in fibrosis, which is essentially characterized by an aberrant composition and overproduction of ECM, is well-documented in several organ systems. Subcutaneous administration of TGF-β elicits rapid dermal fibrosis, while systemic bolus administration results in multiorgan fibrosis (*60*, *81*). Indeed, the various etiologies of fibrotic pathologies in different tissues in which TGF-β has been implicated suggest that it is a significant mediator of this pathological process (*82*).

What is less apparent is how the control of regulation of TGF-β goes awry. Wound healing exemplifies this dilemma. Whereas it is well recognized that TGF-β is a critical mediator of wound healing, it is absent from wound healing in the fetus (*83*). The fact that fetal wound healing reconstitutes the tissue organization without scars while postnatal wound healing results in repair of the tissue but never identical reconstitution suggests that TGF-β may be instrumental in scar formation. This view is supported by studies showing that injection of TGF-β into fetal wounds elicits scarring (*84*) and conversely that adult skin incision wounds injected with TGF-β-neutralizing antibodies healed with less scarring (*85*). Recent studies in pre- and postmenopausal women also support the concept that regulation of TGF-β is critical to repair rather than reconstitution of dermal wounds (*86*). It appears that TGF-β activity is in excess of what is necessary for wound healing under ideal circumstances, such as aseptic surgery. In terms of benefit to the organism, TGF-β may be a part of damage control that has evolved to quickly repair rather than restore tissue integrity. As a consequence it may be poised to overreact, particularly to repeated injury. Likewise, radiation may elicit aberrant activation of TGF-β as part of a damage response whose overall effect is beneficial. The failure of this response to resolve due to a secondary defect in recovery may contribute to adverse responses of normal tissue during radiotherapy of sensitive individuals (*77*, *87*). Since the activity of TGF-β can be manipulated experimentally by administering peptides or antibodies that neutralize activity (*73*) or inhibit activation (*88*), it may be possible to prevent late effects driven by TGF-β without adverse effects in most individuals.

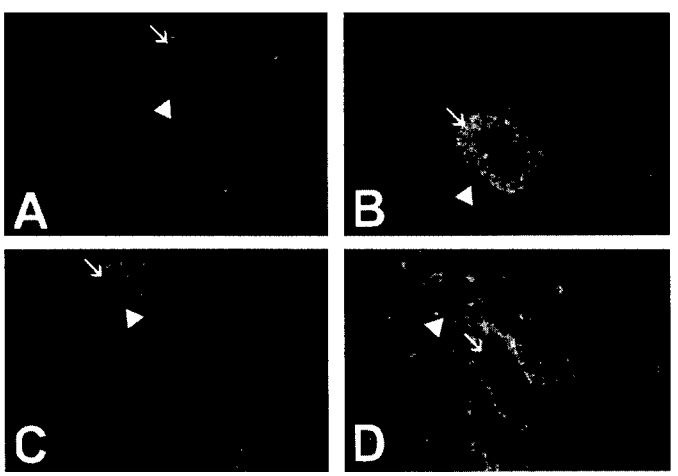

FIG. 3. Relative distribution of tPA (panels A, B) and PAI (panels C, D) in mammary gland from sham-irradiated (panels A, C) and irradiated (panels B, D) animals. Immunoreactive tPA is induced preferentially in the epithelium (arrows) compared to the stroma (arrowheads), while PAI increases in the stroma and adipose. Original magnification was 200×.

INTEGRATED RESPONSES

The response of tissue to radiation is a composite of the results of genetic damage, cell loss and induced gene products. We have used highly sensitive and precise cell biology techniques to map complex patterns of radiation-induced gene expression in the mouse mammary gland that are summarized in Fig. 4. The series of events that occur from 1 h and 4 weeks after whole-body exposure to doses of 0.1 to 5 Gy of ^{60}Co γ radiation demonstrate that irradiated tissues exhibit several general features:

1. The response of tissue to ionizing radiation is global yet innately tissue- and cell type-specific. Thus alterations in protein expression in the mammary gland are distinct from those expressed in liver, and those in the mammary epithelium are distinct from those in the stroma.
2. The response of tissue is evident very soon after radiation exposure.

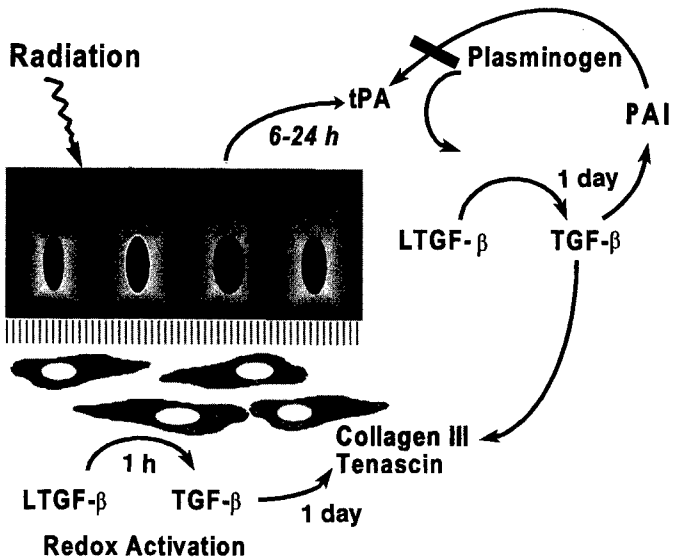

FIG. 4. Schematic representation of the events revealed by immunohistochemistry within the irradiated mammary gland and possible interactions.

3. Radiation-induced changes can be persistent.
4. Some proteins are clearly secondary to others, indicative of a dynamic network.
5. Microenvironment is a target of radiation.
6. The response of tissue can be detected after exposure to low whole-body doses (0.1 Gy).
7. Thresholds for radiation responses are cell type-specific.

An integrated model of the varied and complicated cellular processes governing the response of tissue to radiation exposure could provide valuable indices of susceptibility to radiation and clues for effective intervention.

In wounding, tissue disruption evokes orchestrated programs: bleeding, clot formation and loss of oxygen, all of which elicit global responses. But when injury leaves tissue intact, such as inflammation or exposure to UV or ionizing radiation, what cellular mechanisms record or "sense" the damage? One model holds that there are molecular mechanisms for sensing DNA damage, which is clearly an important determinant of the response of individual cells. However, damaged cells may not be capable of contributing to the repair or reconstitution of tissue and may indeed opt for self-destruction via apoptosis as the altruistic solution for maintaining tissue integrity (*89*). One might postulate that sensors of damage have evolved outside the cell that are capable of registering certain types of damage and producing a signal that will recruit nondamaged cells to facilitate recovery.

Mechanisms

A major mode of ionizing radiation action is the generation of hydroxyl radicals and other reactive oxygen species (*90*). We reasoned that the rapidity of activation of latent TGF-β in irradiated mammary gland suggested an effect attributable to exposure to reactive oxygen species. Activation of latent TGF-β also appears to be concomitant with generation of reactive oxygen species by leukocytes (*91*, *92*) and has been implicated in a number of diseases in which production of reactive oxygen species contributes to the disease state or progression (*93–98*). We postulated that activation of latent TGF-β is similar to the redox sensitivity exhibited by certain transcriptional factors (*99*). This hypothesis was studied in a cell-free model in which recombinant human latent TGF-β was exposed to various mechanisms that generate reactive oxygen species. Release of TGF-β was measured by a biological assay using mink lung epithelial cells to determine the degree of activation under these experimental conditions compared to standard thermal activation. Highly efficient activation was observed as a consequence of exposure to reactive oxygen species generated by metal ion-catalyzed ascorbate oxidation, without any deleterious effect on the activity of TGF-β itself (*99*).

Activation of latent TGF-β *in vivo* that is dependent on reactive oxygen species would be fast, diffuse and independent of protein synthesis, as might be required for the response of tissue to damage or infection. Although this type of activation would be less well controlled compared to cell surface plasmin-mediated activation as described by Rifkin and others (*100–102*), it would have features suited to its role in orchestrating the rapid response of tissue to cellular damage. Release of extracellular TGF-β mediated by reactive oxygen species would expose cells that lack the proteolytic machinery for activation and thus recruit them into the damage response, much as platelet degranulation recruits appropriate cells to the site of a wound. Activation distant from cell surface receptors would favor TGF-β's role as a chemoattractant at sites of inflammation (*103*). Since macrophages, monocytes and polymorphonuclear leukocytes activate latent TGF-β after stimulation (*91*, *92*), activated inflammatory cells are a potential cellular source for reactive oxygen species that could perpetuate latent TGF-β activation after radiation exposure, which would then serve as a gradient for chemotaxis and a stimulus for phenotypic conversion of fibroblasts and monocytes. As such, activation mediated by reactive oxygen species would endow latent TGF-β with the ability to act as a sensor of oxidative stress and, in releasing TGF-β, to signal multiple types of cells to change their phenotype, presumably directed toward recovery of homeostasis.

Consequences

Interestingly, there is mounting evidence that TGF-β itself may signal certain events through the generation of reactive oxygen species (*104–109*). TGF-β induces the production of hydrogen peroxide in bovine endothelial cells (*110*), mouse osteoblastic cells, where it has been shown to be necessary for the transcriptional activation of the *Egr1* gene (*106*), and human lung fibroblasts, where it is generated by the activation of NADH oxidase (*107*). Fanburg and colleagues have suggested that TGF-β in the presence of $FeCl_3$ leads to a pro-oxidant state in bovine endothelial cells (*111*), in part through regulation of Cu,Zn-superoxide

dismutase (*112*) and depletion of glutathione (*113*). Similar suppression of expression of antioxidative enzymes is found in rat hepatocytes (*114*). Recent studies have implicated reactive oxygen species as an important signal for apoptosis induced by TGF-β (*108, 109*). A variety of tumor cells also generate reactive oxygen species (*115*), which could also contribute to nonspecific latent activation of TGF-β leading to immunosuppression or stromal reaction during carcinogenesis (*66*). Thus, in some situations, a self-amplifying cascade could be envisioned in which radiation-induced activation of latent TGF-β results in TGF-β that stimulates nonphagocytic cells to produce reactive oxygen species, which in turn contribute to further activation via the redox sensitivity of latent TGF-β. TGF-β is implicated in a variety of tissue pathologies mediated by reactive oxygen species, including acute respiratory distress syndrome (*93*), atherosclerosis (*97*) and radiogenic fibrosis (*77, 78*), which suggests that oxidative activation of latent TGF-β may be deleterious to tissue in certain disease states leading to chronic production of reactive oxygen species.

IMPLICATIONS AND CONCLUSIONS

The mechanisms by which cells respond to radiation injury and, perhaps more importantly, the way in which these responses alter tissue integrity will ultimately provide the key for manipulating radiation response to benefit therapy. Because there is a wealth of information regarding the role of TGF-β in normal and pathological tissue processes, its involvement in radiation response presents an opportunity to test this general paradigm. The doses in our mammary gland studies were chosen to analyze the cell biology of the irradiated tissue and are insufficient to elicit fibrosis, assuming that mouse mammary gland is a susceptible tissue. Since increased TGF-β mRNA, immunoreactivity and biological activity follow radiation exposure and accompany the development of radiogenic fibrosis in several tissues, the challenge is to determine the consequences of TGF-β activity within the context of the irradiated tissue, to identify the response of irradiated cells to TGF-β, and to determine at what point its activity is irrevocably deleterious. Thus total TGF-β immunoreactivity increases in the irradiated lungs of both fibrosing and nonfibrosing mouse strains, but TGF-β activation occurs only in sensitive mice with lung lesions that are undergoing conversion to fibrosis (*116*). Future studies using neutralization of TGF-β in well-defined models of radiogenic fibrosis will ultimately provide a functional test of the hypothesis that TGF-β activity is an important determinant of the late effects of radiation.

Components of the remodeling of the microenvironment may also contribute to radiation carcinogenesis. It has been demonstrated that irradiated nontransformed cells influence the expression of the transformed phenotype in cell culture, which is evidenced by the decreased frequency of transformation with increased plating density (*117, 118*). Quantitative studies in the mouse and rat mammary gland and rat trachea have demonstrated that the number of cells initiated by either exposure to physical or chemical carcinogens far exceeds the number of tumors that develop (*119–123*). It has been proposed (*124*) and demonstrated experimentally (*125*) that an appropriate microenvironment is necessary for neoplastic progression. This model has been likened to the requirement of seeds (initiated cells) for the appropriate soil (the microenvironment). The multiple controls imposed on cell behavior by the normal microenvironment impede the ability of individual neoplastic cells to flourish. It has been postulated that an essential step in carcinogenesis is to escape negative regulation by normal cells (*126*). Indeed, some of the proteins induced by radiation may be part of a program to suppress the growth of malignant cells. Radiation-induced TGF-β activity may be one such element. The production of active TGF-β by cultures of irradiated rat tracheal epithelial cells has been shown by Terzaghi-Howe to suppress the expression of the transformed phenotype in an *in vitro/in vivo* model of carcinogenesis (*127*). A novel mechanism has been identified by Bauer and colleagues, who have demonstrated that TGF-β-treated normal cells can induce cells transformed by chemicals, viruses or radiation to undergo apoptosis (reviewed in ref. *126*). The elimination of transformed cells by normal cells requires the production of reactive oxygen species as both an intracellular signal and extracellular transducer of the signal that leads to apoptosis. Although this mechanism is described presently only in fibroblast cell models of transformation, Bauer proposes that neoplastic cells must overcome or develop resistance to such signals originating from normal cells to become tumors. It may be that the redox sensitivity of activation of latent TGF-β could augment or perpetuate this stimulus to reduce the persistence of initiated cells in irradiated tissue.

Conversely, disturbances of the microenvironment may provide the stimulus for cells to move partially into the neoplastic pathway (*128*). TGF-β is implicated in transformation of mesenchymal cells in a variety of cell culture models (*129*) and *in vivo* (*130*). As such, the effects of radiation on the microenvironment, via altered gene expression by non-neoplastic cells, may be considered as a third order of carcinogenic action distinct from mutation or mitogenic stimuli (*50*). We propose that radiation disrupts the flow of information between cells and perturbs inhibitions that normal cells effect through cell–cell contact, cell–matrix interactions and production of growth factors. These alterations are postulated to be conducive to neoplastic progression (*50*).

The efficiency of tumor formation might increase if changes in the microenvironment induced by radiation permit initiated cells to elude a normal physiological barrier to expansion. The distinct effects of heavy charged-particle radiation on the basement membrane may contribute to its enhanced carcinogenic potential, which is an order of magnitude greater than that of γ radiation (*131*). Disruption of the basement membrane might remove an obstacle that could otherwise be circumvented only by additional mutations in the initiated cell. For example, the production of transin, a protease that degrades

basement membranes, by radiation-induced benign skin papillomas correlates with their high rate of conversion to malignancy compared to chemically induced tumors (*132*). Another means by which the irradiated microenvironment might influence the development of cancer is if it creates a selective barrier that promotes the expansion of certain clones within the heterogeneous tumor cell population. This mechanism would affect the features or characteristics of radiogenic tumors. Radiation-induced TGF-β activity may fall into this category since, although normal and premalignant cells are profoundly inhibited by TGF-β, resistance to TGF-β is a common cancer cell phenotype. Thus, radiation-induced TGF-β activity could promote the expansion of such phenotypes (*133*). The impact of the irradiated microenvironment on neoplastic progression is currently under investigation in this laboratory. Preliminary data indicate that p53-mutant mammary epithelial cells are preferentially tumorigenic in the irradiated mammary gland.[2]

As our understanding of cell regulation expands to include the critical role of microenvironment, the unit of function is no longer single cells. This perspective is likely to be fruitful for understanding tissue responses to radiation. Radiation-induced extracellular signaling has important ramifications for understanding the restitution of tissue integrity and, conversely, persistent dysfunction (*134*). Future studies may be able to use early events associated with radiation-induced microenvironments to predict the long-term consequences in individuals. Furthermore, extracellular signaling may be a suitable target for manipulation directed toward dissociating events leading to tissue recovery from those that progress to pathology.

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